The Role of BIN1 in the Regulation of Cell Proliferation, Apoptosis and Tumor Formation in Cutaneous T-Cell Lymphoma

by

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Abstract

Cutaneous T-cell lymphomas (CTCLs) represent a group of lymphoproliferative disorders characterized by homing of malignant T-cells to the skin’s surface. There are two main types of CTCL: Mycosis Fungoides (MF) and Sezary Syndrome (SS). We have demonstrated that expression of the Abelson helper integration site-1 (AHI-1) oncogene is significantly increased in CD4+CD7- cells from SS patients. Bridging integrator 1 (BIN1) has been identified by microarray analysis of CTCL cells as a candidate gene involved in AHI-1-mediated lymphomagenesis. Interestingly, BIN1 expression is significantly reduced in SS patient samples. However, the role of BIN1 and its molecular connection to AHI-1 in lymphomagenesis remains unexplored.

I extensively investigated the role of key BIN1 isoforms in primary and CTCL cell line model systems both in vitro and in vivo. I demonstrated that overexpression/restored expression of BIN1 isoforms has strong anti-proliferative and pro-apoptotic roles in CTCL cells in vitro, and significantly inhibits the tumorigenic activity of these cells in vivo. The pro-apoptotic role of BIN1 in CTCL cells occurs through downregulation of c-FLIP, a critical inhibitor of Fas/FasL-mediated apoptosis. I also observed significant reduction and increase in BIN1 and c-FLIP transcripts in primary CTCL samples, respectively. Interestingly, high BIN1 and low c-FLIP transcripts correlated with better survival rate in SS patients. Thus, BIN1 deficiency may play an important role in CTCL pathogenesis by causing apoptosis resistance.

Furthermore, I explored potential mechanisms by which AHI-1 leads to downregulation of BIN1, by (1) examining if AHI-1 physically interacts with BIN1; and (2) determining if AHI-1 alters transcription of BIN1 by changing the methylation status of the BIN1 promoter. These experiments did not yield direct evidence of these two potential mechanisms of AHI-1’s role in
BIN1 suppression. Thus, the mechanism by which AHI-1 regulates BIN1 remains unknown. Nevertheless, several potential BIN1 interacting proteins were uncovered in CTCL cells, including α/β-tubulin and β-actin.

Overall, this study provides the first evidence of strong tumor suppressor activity of BIN1 in CTCL. It points to the loss of BIN1 and subsequent upregulation of c-FLIP as an important mechanism to induce apoptosis resistance in CTCL cells, and identifies BIN1 and c-FLIP as potential CTCL therapeutic targets.
Preface

All experiments were conducted by Sharmin Esmailzadeh, except for the parts stated below. I designed the experiments presented in this dissertation, analyzed and interpreted all the data, composed and edited the thesis, under the supervision of Dr. Xiaoyan Jiang at the Terry Fox Laboratory, BC Cancer Research Centre.

Some of the sections presented in Chapter 1, including Figures 1.5 and 1.6 have been published in a first-author review paper. Esmailzadeh S and Jiang X (2011) “AHI-1: a novel signaling protein and potential therapeutic target in human leukemia and brain disorders.” Oncotarget. 2(12):918-34. Some of the results presented in Chapter 1, including Figure 1.8 have been published in a middle-author publication. Kennah E, Ringrose A, Zhou LL, Esmailzadeh S, Qian H, Su MW, Zhou Y, Jiang X. (2009) “Identification of tyrosine kinase, HCK, and tumor suppressor, BIN1, as potential mediators of AHI-1 oncogene in primary and transformed CTCL cells.” Blood. 113(19):4646-55.

A version of Chapter 3 has been submitted for publication. Esmailzadeh S, Su M, Zhou Y, Jiang X. “BIN1 tumor suppressor regulates Fas/Fas ligand–mediated apoptosis through c-FLIP in cutaneous T-cell lymphoma.” I performed all the experiments, analyzed the data, generated all the figures and wrote the manuscript. The RNA samples from patients with mycosis fungoides (MF) and Sezary syndrome (SS) were extracted and provided by Ming-Wan Su under the supervision of Dr. Youwen Zhou from the Department of Dermatology, University of British Columbia. All the other authors had intellectual input in the study and manuscript writing.

The mass spectrometry analysis presented in Chapter 4, was conducted in collaboration with Dr. Gregg Morin at the Michael Smith Genome Sciences Centre, BC Cancer Agency. In
addition, the bisulfite sequencing presented in Chapter 4, was conducted in collaboration with Dr. Dixie Mager at the Terry Fox Laboratory, BC Cancer Research Centre. The studies in this thesis performed with primary samples from patients with MF and SS were approved by the University of British Columbia-Clinical Research Ethics Board, certificate number H12-02653. Mice were housed in the Animal Resource Centre (ARC) with the assistance of ARC staff. Mouse studies were performed under UBC Animal Care protocol A11-0005 (Dr. Xiaoyan Jiang). The use of biohazardous materials and chemicals was approved by the UBC Biosafety Committee, certificate number B10-0165. The use of radioisotopes was approved by the Canadian Nuclear Safety Commission, license number 06074-16-17.8.

Dr. Xiaoyan Jiang contributed to the experimental design, data interpretation, and editing of the dissertation.
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List of Abbreviations

a.k.a. = Also known as
ACAD = Activated cell autonomous death
AD = Alzheimer’s disease
AHI-1 = Abelson helper integration site-1
AICD = Activation-induced cell death
A-MuLV = Abelson-murine leukemia virus
APC = Antigen presenting cell
BAR = Bin/Amphiphysin/RVS
BCL-2 = B-cell lymphoma-2
BCR = B-cell receptor
BH = BCL-2 homology
BIN1 = Bridging integrator 1
BM = Bone marrow
BSA = Bovine serum albumin
C = Cytoplasmic
CCR4 = chemokine (C-C motif) receptor 4
CFC = Colony-forming cell
c-FLIP = Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein
CGI = CpG island
CHIP = Chromatin immunoprecipitation
CLA = Cutaneous lymphocyte antigen
CLP = Common lymphoid progenitor
CML = Chronic myeloid leukemia
CMP = Common myeloid progenitor
CNM = Centronuclear myopathy
CNS = Central nervous system
CTCL = Cutaneous T-cell lymphoma
D = Diversity
DC = Dendritic cell
DED = Death effector domains
DISC = Death-inducing signaling complex
DMEM = Dulbecco's Modified Eagle Medium
DN = Double-negative
DP = Double-positive
E1A = Early-region 1A
ECP = Extracorporeal photopheresis
EORTC = European Organization for Research and Treatment
FACS = Fluorescein-activated cell sorter
FADD = Fas-associated death domain protein
FasL = Fas ligand
FBS = Fetal bovine serum
GAPDH = Glyceraldehydes-3-phosphate-dehydrogenase
H&E = Hematoxylin and eosin
HIV = Human Immunodeficiency Virus
HSC = Hematopoietic Stem Cell
ICAM-1 = Intercellular adhesion molecule-1
IF = Immunofluorescence
IFN-γ = Interferon gamma
IgG = Immunoglobulin G
IHC = Immunohistochemistry
IL = Interleukin
IP = Immunoprecipitation
J = Joining
JS = Joubert syndrome
JSRD = Joubert syndrome related disorders
Kb = Kilobase
Kd = kilodalton
LC = Langerhans cell
LFA-1 = Lymphocyte function-associated-1
MBD = MYC binding domain
MEF = Mouse embryo fibroblasts
MF = Mycosis Fungoides
MHC = Major histocompatibility complex
miRNAs = microRNAs
MMD = Myotonic muscular dystrophy
N = Nuclear
NK = Natural killer
NOD = Non-obese diabetic
NTS = Neural tissue specific
PB = peripheral blood
PBS = Phosphate buffered saline
PI = Propidium iodide
PIC = Protease inhibitor cocktail
PMSF = Phenylmethylsulfonyl fluoride
PS = Phosphatidylserine
PSB = Phosphorylation solubilization buffer
qRT-PCR = Quantitative RT-PCR
RBC = Red blood cell
RPMI = Rosewell Park Memorial Institute
RT = Rhabdoid tumors
RT = Room temperature
RT-PCR = Reverse transcription polymerase chain reaction
SCID = Severe-combined immunodeficiency
SD = Standard deviation
SEM = Standard error of the mean
SH3 = Src homology 3
siRNA = Small interfering RNA
SP = Single positive
SS = Sezary syndrome
TBST = Tris-buffered saline Tween 20
TCR = T-cell receptor
TKI = Tyrosine kinase inhibitor

TNF-α = Tumor necrosis factor alpha

Treg = T-regulatory

TUNEL = Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate end labeling

UV = Ultraviolet

V = Variable

WBC = White blood cell

WHO = World Health Organization

μCi = Microcurie

μg = Microgram

μL = Microlitre

μm = Micrometre
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I dedicate this thesis

to

my beautiful parents, for their unconditional love and endless support

and

Kiyan, for his limitless love
Chapter 1: Introduction

1.1 Overview of Hematopoiesis

Hematopoiesis is a continuous, regulated process of generation, development and specialization of blood cells. Blood contains various types of cells with different life-spans and different functions, which are all derived from multipotent hematopoietic stem cells (HSCs) (Till et al. 1980; Chao et al. 2008; Dick 2008; Notta et al. 2011). HSCs can be found in adult bone marrow (BM), peripheral blood (PB), and umbilical cord blood. Under normal conditions, HSCs are primarily found in the BM in the ratio of about 1:1,000 nucleated blood cells and only small numbers of them can be detected in the PB (Till et al. 1980; Rodak et al. 2007; Dick 2008). Two main characteristics of HSCs are self-renewal and multipotency. Self-renewal is the ability to produce more HSCs without differentiation, while multipotency is the ability to differentiate into all hematopoietic lineages and functional blood cells (Osawa et al. 1996; Bryder et al. 2006; Alberts 2008; Chao et al. 2008). Thus, by balancing self-renewal and differentiation, the HSC pool is maintained while differentiated progenitors are produced throughout an individual’s life. HSCs sit at the top of the hierarchy of multilineage progenitors that gradually become committed to several or single lineages and finally result in differentiation and production of mature blood cells (Figure 1.1) (Till et al. 1980; Osawa et al. 1996; Bryder et al. 2006; Chao et al. 2008). One of the early commitment steps in HSC differentiation is the diversification of the lymphoid and myeloid cell lineages. The common lymphoid progenitors (CLPs) give rise to all types of lymphocytes (B-cells, T-cells and natural killer cells), whereas the common myeloid progenitors (CMPs) differentiate into all classes of myeloid cells (granulocytes, monocytes/macrophages, erythrocytes and thrombocytes), with no or extensively low levels of B-cell potential (Abramson
et al. 1977; Till et al. 1980; Akashi et al. 2000; Kondo 2010). Multiple divisions of committed progenitors lead to the production of large numbers of mature blood cells. The multitiered hematopoietic differentiation scheme has the benefit of putting very little proliferative pressure on HSCs, which primarily reside in the G0 phase of the cell cycle and divide infrequently. This minimal proliferative pressure on HSCs has the advantage of reducing the rate of potential mutations following the DNA replication and cell division, thus contributing to the integrity and longevity required of these cells (Bradford et al. 1997; Bryder et al. 2006). Furthermore, the hierarchical nature of the hematopoietic system also significantly decreases the risk of developing leukemia and lymphoma (Wang & Dick 2005). Due to the high proliferation rate of progenitors, mutations are more likely to arise in these cells. However, due to the limited life span of the progenitor cells, they will likely undergo terminal differentiation or apoptosis before the required mutations for developing leukemia can accumulate (Wang & Dick 2005; Becker & Jordan 2011; Visvader & Lindeman 2012). Yet, mutations in HSCs can alter the self-renewal capacity or proliferation rate of HSCs or more committed progenitors that are responsible for the development and long-term maintenance of human leukemia (Wang & Dick 2005; Dick 2008; Visvader & Lindeman 2012).

In general, mature PB cells can be classified into two major groups of red blood cells (RBCs, also known as (a.k.a.) erythrocytes) and white blood cells (WBCs, a.k.a. leukocytes) (Alberts 2008). RBCs function within the blood vessels and are responsible for transporting oxygen and carbon dioxide bound to hemoglobin. WBCs, unlike RBCs, migrate through the walls of small blood vessels into tissues and have different functions, including combating infections, phagocytosis, and digesting debris (Ellsworth et al. 1995; Alberts 2008; Cashen et al. 2012). Furthermore, blood contains platelets (a.k.a. thrombocytes), which are detached
cytoplasmic fragments released from large cells called megakaryocytes and are essential factors in the process of blood clotting (Gawaz et al. 2005; Alberts 2008; Cashen et al. 2012).

All RBCs belong to a single category and follow the same developmental pathway, and the same is true for platelets. However, WBCs are divided into three distinct categories, namely: granulocytes, monocytes, and lymphocytes (Till et al. 1980; Dick 2008; Cashen et al. 2012). Granulocytes contain many lysosomes and secretory vesicles and are divided into three sub-classes based on the morphology and staining pattern of these organelles: neutrophils, basophils, and eosinophils (Rodak 2002; Cashen et al. 2012). Neutrophils have multi-lobed nuclei and are able to phagocytose and destroy microorganisms. Basophils contain bi-lobed nuclei and large cytoplasmic granules and mediate inflammatory responses by secreting histamine. Eosinophils also have multi-lobed nuclei (usually 2 to 4 lobes) and are responsible for destroying parasites and controlling mechanisms associated with allergic inflammatory responses (Rodak 2002; Cashen et al. 2012; De Kleer et al. 2014).

The second major group of WBCs contain monocytes. After leaving the bloodstream, monocytes differentiate into macrophages, which are the main phagocytes in the body along with neutrophils (Alberts 2008; De Kleer et al. 2014). Macrophages recognize and eliminate senescent, necrotic, and damaged cells in different tissues. They also have the unique capability of ingesting large microorganisms such as protozoa (Rodak 2002; Alberts 2008; Cashen et al. 2012). Monocytes can also differentiate into dendritic cells (DCs), which mainly present foreign antigens to lymphocytes and trigger an immune response. Langerhans cells (LC), for example, are DCs of the epidermis, which ingest foreign antigens and present them to lymphocytes in lymph nodes (Rodak 2002; Cashen et al. 2012; De Kleer et al. 2014).
The third major group of WBCs contain lymphocytes that are involved in immune responses and include B-cells, T-cells, and natural killer (NK) cells (Alam & Gorska 2003; Larosa & Orange 2008). There are two broad groups of immune responses: antibody responses and cell-mediated responses, which are carried out by B-cells and T-cells, respectively (Alberts 2008). B-cells are distinguished from other lymphocytes by the presence of specific transmembrane receptor proteins on their surface, called B-cell receptors (BCRs). A specialized BCR allows a B-cell to detect and bind to a specific antigen. This binding, leads to activation of the B-cell, as well as secretion of large amounts of soluble antibody specific to the presenting antigen (Alam & Gorska 2003; Larosa & Orange 2008; Treanor 2012). The soluble antibodies can circulate in the bloodstream and can act far away by binding to the particular antigens that triggered their initial production (Alam & Gorska 2003; Larosa & Orange 2008; Treanor 2012). Binding of antibodies can either inactivate microbial toxins or viruses by preventing them from binding to receptors on host cells, or by making them easier targets for phagocytes to ingest (Alberts 2008). T-cells are another group of lymphocytes, characterized by the presence of T-cell receptors (TCRs) on their surface (Nijkamp et al. 2011). Similar to B-cells, T-cell responses are also antigen-specific. However, T-cell and B-cell responses are different in at least two ways. First, T-cells can only recognize fragments of antigens that have been partially degraded by antigen-presenting cells, whereas B-cells can detect intact antigens (Alam & Gorska 2003; Larosa & Orange 2008). The second difference is that TCRs on T-cells, unlike antibodies produced by B-cells, exist only as membrane-bound molecules and are not secreted. Thus, T-cell responses act only at short range through direct interaction with target cells, either within a secondary lymphoid organ or at a site of infection (Alam & Gorska 2003; Larosa & Orange 2008). The function and development of T-cells are further discussed in more detail in Section
1.2. In addition to B- and T-cells, NK cells are capable of eliminating virus-infected cells and some types of tumor cells (Trinchieri 1989; Vivier et al. 2008). In general, all hematopoietic cell types other than lymphocytes are referred to as myeloid cells. Deregulation of the hematopoietic differentiation pathways may lead to a drastic change in the number of mature blood cells in the body, which is usually an indication of an underlying disease or disorder (Cashen et al. 2012).
Figure 1.1: Hierarchy of the hematopoietic differentiation

The HSC has the ability to self-renew and generate more stem cells or to divide and further differentiate into progenitors that can generate a more restricted set of cells. LT-HSC = long-term repopulating HSC; ST-HSC = short-term repopulating HSC; MPP = multipotent progenitor; CMP = common myeloid progenitor; CLP = common lymphoid progenitor; MEP = megakaryocyte/erythroid progenitor; GMP = granulocyte–macrophage progenitor.
1.2 T-cell Biology

To gain better insights into specific perturbations occurring in T-cell malignancies, such as cutaneous T-cell lymphoma, it is initially important to understand the developmental paths and functions of normal T-cells. The human immune system is a network of cells, tissues, and organs that work together to defend the host from infection and disease (Alam & Gorska 2003). There are two separate, but intertwined, branches of immune response called innate and adaptive responses. The innate immune system acts as the first line of defense, its responses to invading pathogens are generic and non-specific, and it does not confer long-lasting immunity to the host (Alam & Gorska 2003; Alberts 2008; Larosa & Orange 2008). Compared to the innate immunity, the adaptive immune system is a more sophisticated defense mechanism with the ability to generate antigen-specific responses and develop immunological memory (Alam & Gorska 2003; Alberts 2008; Larosa & Orange 2008). Lymphocytes are the key components of the immune system and include T-cells, B-cells, and NK cells. NK cells are the components of the innate immune system and their functions include perforin-dependent cytotoxicity, cytokine production, and induction of co-stimulatory molecules and thus, facilitating the adaptive immunity (Alam & Gorska 2003; Alberts 2008). T-cells and B-cells are major players of the adaptive immune system. In general, T-cells are instrumental in eradicating intracellular pathogens, either directly or by recruiting other leukocytes, whereas B-cells defend against extracellular pathogens though antibody production (Alam & Gorska 2003; Larosa & Orange 2008).
1.2.1 T-cell Function

The keystone of the adaptive immune system is the interaction between T-cells and antigen presenting cells (APCs). TCRs only recognize antigens in the form of short linear peptides of 10-12 amino acids long, which are presented by APCs (Alam & Gorska 2003; Alberts 2008). APCs possess major histocompatibility complex (MHC) molecules on their surface and have the ability to process antigens into short linear peptides. These peptides are then presented in the MHC grooves of APCs and are subsequently recognized by the cognate TCRs (Alam & Gorska 2003). MHCs are divided into two major classes: MHC class I and MHC class II. MHC class I molecules are found on all nucleated cells, recognized by CD8\(^{+}\) T-cells and present peptides derived from intracellular proteins, while MHC class II molecules are only expressed on typical APCs (e.g. dendritic cells, B-cells, and macrophages), are recognized by CD4\(^{+}\) T-cells and present peptides from extracellular proteins (Alam & Gorska 2003; Nijkamp et al. 2011). The interaction between the TCR and the MHC-antigen complex provides an antigen recognition step and initiates the T-cell activation process, also known as “T-cell priming” (Alam & Gorska 2003; Nijkamp et al. 2011). Other factors such as co-receptors (CD4 and CD8), co-stimulatory molecules and accessory molecules are also required for enhancing and maintaining the T-cell activation (Nijkamp et al. 2011). Co-receptors such as CD4 and CD8 are important in assisting the TCR signal through recruiting the tyrosine kinase LCK and stabilizing the interaction between the TCR and MHC-antigen complex, respectively (Nijkamp et al. 2011). Furthermore, the co-stimulatory molecules such as CD28 and lymphocyte function-associated-1 (LFA-1), which bind to their respective ligands, B7 and intercellular adhesion molecule-1 (ICAM-1) on APCs, are involved in enhancing and strengthening the interaction between T-cells and APCs and are crucial for sustaining signal transductions (Larosa & Orange 2008; Nijkamp et al. 2011).
al. 2011) (Figure 1.2). Signaling through both the TCR and a co-stimulatory receptor is pivotal for complete activation of T-cells and further production of interleukin (IL)-2, which is an important cytokine for the clonal expansion of the activated antigen-specific T-cells. The clonal expansion can temporarily increase the number of antigen-specific T-cells from < 0.001% to >30% of the total T-cell population (Alam & Gorska 2003). However, incomplete activation of T-cells in the absence of co-stimulatory signals leads to inhibition of IL-2 production and induces T-cell anergy, a tolerance mechanism important in the prevention of self-reactive T-cells and autoimmunity (Alam & Gorska 2003; Larosa & Orange 2008).

There are two main types of mature T-cells in the blood: CD4+ helper T-cells and CD8+ cytotoxic T-cells. Naïve CD4+ T-cells have not yet encountered their cognate antigen within the periphery and thus have not been activated (Nijkamp et al. 2011). However, upon activation, these naïve CD4+ T-cells can be further divided into distinct subsets based on their cytokine profiles, namely Th1, Th2, Th17, and T-regulatory cells (Tregs) (Alam & Gorska 2003; Zhu & Paul 2008; Nijkamp et al. 2011). Th1 cells activate macrophages by producing interferon gamma (IFN-γ) and are responsible for eliminating intracellular pathogens (Larosa & Orange 2008). Th2 cells facilitate B-cell antibody responses by producing the IL-4, IL-5, and IL-13 cytokines and are important in extracellular pathogen clearance (Larosa & Orange 2008). The more recently emerged Th17 subset plays a role in proliferation and recruitment of neutrophils by production of IL-17, IL-6, and tumor necrosis factor alpha (TNF-α) (Larosa & Orange 2008). Finally, the regulatory subset Treg constitutively expresses the IL-2 receptor and the transcription factor FOXP3 and has a major role in suppressing T-cell responses and subsequently inhibiting autoimmunity (Larosa & Orange 2008).
Naïve CD8+ cells develop into activated cytotoxic T-cells upon recognition of foreign antigens in the MHC class I grooves on APCs (Nijkamp et al. 2011). CD8+ T-cells have two distinct mechanisms of cytotoxicity and mediating cell death, namely Ca2+-dependent perforin/granzyme-mediated apoptosis, and Ca2+-independent Fas/Fas ligand (FasL)-mediated apoptosis (Alam & Gorska 2003; Nijkamp et al. 2011). Activated CD8+ T-cells have the ability to release lytic granules that include granzymes and perforin. Granzymes are effector molecules that can enter the target cells through plasma membrane pores formed by perforin and are able to induce apoptosis through both caspase-dependent and -independent pathways (Nijkamp et al. 2011). In addition to the production of lytic granules, activated CD8+ T-cells can also induce cell death through the engagement of their FasL with Fas receptor on target cells, which leads to activation of the downstream caspase cascade and apoptosis (Nijkamp et al. 2011).

After successful elimination of foreign antigens, the number of activated CD4+ and CD8+ T-cells shrinks as rapidly as it expanded (Alam & Gorska 2003). For a rapid reduction in cell numbers, activated T-cells employ several self-limited mechanisms including stimulation of inhibitory receptors coupled with additional mechanisms called “activated cell autonomous death” (ACAD) and “activation-induced cell death” (AICD) (Alam & Gorska 2003; Nijkamp et al. 2011), which are described in more detail in Sections 1.2.3.1 and 1.2.3.2. However, several antigen-specific CD4+ and CD8+ T-cells manage to persist after the “shrinkage” phase of the immune response and form the pool of memory T-cells. These memory cells are long-lived T-cells that are dependent on the presence of IL-7 and IL-15 for survival. Furthermore, in the event of a second attack with the same pathogen, these memory T-cells are able to mount a more vigorous and accelerated response, which leads to faster clearance of the foreign antigens (Alam & Gorska 2003; Larosa & Orange 2008; Nijkamp et al. 2011). Memory T-cells display the
CD45RO marker and are divided into two major populations called effector and central memory T-cells. The effector memory T-cells lack the expression of CCR7 and L-selectin, circulate the peripheral tissue, and are able to execute effector functions instantly. In contrast, the central memory T-cells reside in lymphoid organs due to the expression of CCR7 and L-selectin, and upon antigen stimulation, they start proliferating rapidly and differentiating into effector memory T-cells (Alam & Gorska 2003; Larosa & Orange 2008; Nijkamp et al. 2011). Therefore, the central memory T-cells act as the back-up reservoir for the effector memory T-cell population.

In conclusion, different T-cell subsets coordinate an efficient adaptive immune response either by a direct effect on target cells or by secreting cytokines and recruiting other leukocytes. Therefore, the role of each subset is significantly important for developing an accurate response and distinguishing “self” from “non-self”. Consequently, T-cell development is a highly regulated, multi-step process with stringent checkpoints to ensure that only functionally normal cells are allowed to fully mature.
Figure 1.2: Schematic view of stimulatory signals required for complete activation of T-cells

The first signal (1) is the interaction between the TCR and its cognate antigen (red diamond) present on the MHC molecule on the surface of the APC. This interaction provides an antigen recognition step and initiates the T-cell activation process. The second co-stimulatory signal (2) is required for the complete T-cell activation, such as the interaction between CD28 and its respective ligand B7 on the surface of APC. This interaction enhances and strengthens the interaction between T-cells and APCs and is crucial for sustaining subsequent signal transductions.
1.2.2 T-cell Development

The generation of T-cells is unique since they are the only hematopoietic cells that undergo development in the thymus instead of the bone marrow (Rothenberg & Taghon 2005; Nijkamp et al. 2011). A fully differentiated thymus contains several lobules, each with an inner medulla and a surrounding outer cortex. The junction between the medulla and the cortex is where the blood-borne precursors from the bone marrow enter the thymus (Ciofani & Zuniga-Pflucker 2007). These progenitors are mostly “double-negative” (DN) due to lack of expression of the two cell surface markers associated with mature T-cells, namely CD4 and CD8 (Hayday & Pennington 2007). DN cells are located in the cortex and undergo four distinct developmental stages, DN1 to DN4 (Figure 1.3). The TCR rearrangement initiates during the DN2 stage (Ciofani & Zuniga-Pflucker 2007; Hayday & Pennington 2007). TCR consists of the CD3 complex and four gene segments, α, β, γ and δ, which their diverse pattern of rearrangements in the thymus leads to the development of two distinct lineages of T-cells, namely αβ and γδ T-cells (Pennington et al. 2005; Lauritsen et al. 2006). The αβ T-cells are mainly involved in MHC-dependent antigen-specific immune responses, whereas the γδ T-cells are mostly engaged in MHC-independent responses and may potentially regulate and complement the role of αβ T-cells (Pennington et al. 2005; Lauritsen et al. 2006).

The somatic rearrangement of the α, β, γ and δ chains results in the vast repertoire of unique TCRs. As members of the immunoglobulin family, each TCR chain contains two or three gene segments called variable (V), diversity (D), and joining (J), and the recombination of these segments with the random addition of nucleotides at segment connections, determine the TCR repertoire (Alam & Gorska 2003; Larosa & Orange 2008). Maintaining a translational reading frame is essential to generate a functional TCR. However, since the joining events of
V(D)J recombination are random, only one in three attempts are successful and these cells continue to survive and go through the next stages of development (Lauritsen et al. 2006; Larosa & Orange 2008). Although, it is thought that both γδ and precursors to αβ T-cells are derived from DN cells, however, the precise point of their divergence remains to be established (Pennington et al. 2005). DN thymocytes that express the in-frame γδ-TCR are able to differentiate along the γδ-lineage pathway between DN2 and DN3 stages and leave the thymus while remaining DN (Lauritsen et al. 2006). In contrast, DN thymocytes with successful β loci recombination during the DN4 stage, pairs with the surrogate α receptor and finally differentiate along the αβ T-cell lineage, which is further accompanied by a transition from DN to the CD4⁺CD8⁺ double-positive (DP) stage (Lauritsen et al. 2006). At this point, DP cells undergo positive selection and those that bind to MHC molecules with sufficient affinity are able to survive (Lauritsen et al. 2006). In the next stage, sufficient affinity to either MHC class I or MHC class II molecules further differentiates the DP cells into single positive (SP) CD4⁺ or CD8⁺ mature T-cells, respectively, while they localize back in the medulla (Lauritsen et al. 2006). The final step of developing the functionally normal mature T-cell is called the “negative selection”, where the majority of SP cells that interact too strongly with self-antigens are eliminated by apoptosis (Lauritsen et al. 2006). The majority of mature T-cells in peripheral blood consist of the SP αβ lineage, whereas the DN cells with γδ lineage comprise only 1-5% of circulating cells with their majority located in epithelial tissues (Pennington et al. 2005; Lauritsen et al. 2006). These rigorous positive and negative selection stages ensure the production of functionally normal mature T-cells capable of identifying and eliminating foreign antigens, while avoiding interaction with self-antigens, thus inhibiting autoimmunity.
**Figure 1.3: Schematic view of the T-cell development within the thymus**

DN progenitor cells migrate to the thymus from the bone marrow. These cells undergo four distinct developmental stages, DN1 to DN4. The first differentiation branch begins with TCR rearrangement during DN2 stage and further proceeds to DN3 stage where the γδ and αβ lineages diverge. The majority of the γδ T-cells leave the thymus as DN cells, whereas αβ T-cells pass through a DP stage before further differentiation into either CD4+ or CD8+ T-cells. CD4+ cells further mature into Th1, Th2, Th17, or Tregs. Over the course of differentiation in the thymus, the cells migrate out towards the cortex and then back to the medulla. Daggers (†) mark cells undergoing apoptosis which fail to pass developmental checkpoints.
1.2.3 T-cell Homeostasis

Stimulation and control of apoptosis in lymphocytes is more complex in comparison to other cells, as lymphocytes can clonally and rapidly expand and migrate to different parts of the body. Moreover, lymphocytes may cause autoimmunity or lymphoproliferative disorders if they become activated against self-antigens or stay in circulation after an immune response is over (Meech et al. 2001; Alam & Gorska 2003). Therefore, to maintain homeostasis, the clonally expanded activated T-cells must be eliminated once the invading antigen has been eradicated. Hence, additional strategies have evolved in lymphocytes to further control their apoptosis. In T-cells, their activation is programmed to be self-limited through multiple mechanisms (Alam & Gorska 2003; Krammer et al. 2007). One mechanism is through the gradual upregulation and stimulation of inhibitory receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD1), which act as brakes on the T-cell response and provide negative feedback to activated T-cells (Krummel & Allison 1995; Latchman et al. 2001; Alam & Gorska 2003). Despite their importance, however, the mechanisms of action of these inhibitory receptors are poorly understood. There are two other important mechanisms to maintain the T-cell homeostasis, termed “activated cell autonomous death” (ACAD) and “activation-induced cell death” (AICD) pathways, which are explained in detail below.

1.2.3.1 Activated Cell Autonomous Death (ACAD)

ACAD which is also referred to as “death by neglect” or “death by cytokine withdrawal” occurs through cytokine deprivation and absence of appropriate survival signals upon antigen clearance (Hildeman et al. 2002; Krammer et al. 2007). This pathway predominantly relies on the B-cell lymphoma-2 (BCL-2) family of proteins and intrinsic mitochondrial apoptosis.
pathway. The members of the BCL-2 family have either anti-apoptotic or pro-apoptotic activities and share one or more of the BCL-2 homology (BH) domains (BH1 to BH4). They are classically grouped into three classes: the first class inhibits apoptosis and shares the BH1–4 homology domains (e.g. BCL-2, BCL-XL); the second class is pro-apoptotic and shares BH1–3 domains (e.g. BAX and BAK); and the third is a large family of pro-apoptotic BH3-only proteins (e.g. BIM and PUMA) (Hildeman et al. 2002; Youle & Strasser 2008). The balance between pro- and anti-apoptotic family members plays an essential role in the regulation of the intrinsic apoptosis pathway. This is achieved by controlling the mitochondrial membrane permeability and the release of the pro-apoptotic factors including cytochrome c, which leads to the downstream activation of caspase 9 and eventual cell death (Hildeman et al. 2002; Youle & Strasser 2008). During ACAD, the BCL-2 levels within activated T-cells are significantly decreased compared with resting T-cells. One of the important mechanisms for BCL-2 downregulation is lack of survival cytokines that sustain BCL-2 levels including members of the IL-2 family (Duke & Cohen 1986; Marrack et al. 1999). Furthermore, reactive oxygen species and PTEN expression have also shown to be important in BCL-2 downregulation within activated T-cells upon antigen clearance (Hildeman et al. 1999; Suzuki et al. 2001). Thus, downregulation of BCL-2 in activated T-cells may tilt the balance in favor of pro-apoptotic BCL-2 family members. Furthermore, it has been shown that the overexpression of two pro-apoptotic BCL-2 members, BIM and PUMA, is also essential for ACAD. The expression level of BIM and PUMA is upregulated after cytokine deprivation which eventually leads to cytochrome c release and T-cell apoptosis (Krammer et al. 2007).
1.2.3.2 Activation-induced Cell Death (AICD)

AICD is another important mechanism in maintaining the T-cell homeostasis in the periphery. AICD is triggered by TCR re-stimulation of already activated T-cells and predominantly acts through the extrinsic apoptosis pathways (Krammer et al. 2007; Brenner et al. 2008). The term AICD was first used in 1989 after it was demonstrated that T-cell hybridomas undergo apoptosis upon stimulation of their CD3 molecules (Shi et al. 1989). Later on, further studies have demonstrated the importance of the extrinsic Fas-mediated apoptosis pathway in AICD (Dhein et al. 1995; Ju et al. 1995; Sytwu et al. 1996; Gorak-Stolinska et al. 2001). In summary, ligation of the Fas receptor with its ligand (FasL) results in subsequent activation of the caspase cascade including caspase 8 and caspase 3, which eventually leads to apoptosis (Nagata 1999; Meech et al. 2001; Krammer et al. 2007). Moreover, the cellular Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein (c-FLIP) is a well-known inhibitor of extrinsic apoptosis pathways, which can block the activation of caspase 8 and inhibit the cell-death-receptor-induced apoptosis (Thome & Tschopp 2001; Krammer et al. 2007). Repetitive TCR stimulation leads to the co-expression of Fas and FasL on the same activated T-cell, as well as reduction in the c-FLIP expression, resulting in cell death via caspase 8 activation (Brunner et al. 1995; Ju et al. 1995). It has been suggested that the activation of IL-2 potentiates AICD via Fas-mediated apoptosis through regulating the expression levels of FasL and c-FLIP. High expression level of IL-2 is required to suppress the transcription of c-FLIP and enhances the expression level of FasL. Therefore, early in the immune response, when IL-2 levels are low, freshly activated T-cells express high levels of c-FLIP and low levels of FasL. Hence, these cells are resistant to Fas signaling. Conversely, at the height of an immune response, activated T-cell numbers and concentration of IL-2 are maximally present, leading to
the upregulation of FasL, downregulation of c-FLIP and subsequent elimination of these T-cells through Fas signaling (Kneitz et al. 1995; Latinis et al. 1997; Refaeli et al. 1998). In addition to the caspase-dependent mechanisms stated above, other apoptotic pathways including caspase-independent pathways have also been described in T-cell homeostasis (Devadas et al. 2006). Any disruption of these mechanisms may have severe consequences including autoimmunity or the development of T-cell malignancies, such as cutaneous T-cell lymphoma.

1.3 Cutaneous T-cell Lymphoma (CTCL)

CTCL represents a heterogeneous group of non-Hodgkin lymphomas characterized by an initial infiltration of the skin with clonally-derived malignant T-cells (Assaf et al. 2004; Klemke et al. 2006; Booken et al. 2008; Li et al. 2012). CTCL is the most common type of cutaneous lymphomas with the incidence rate of about 6 cases per one million people and accordingly about 3,000 new cases annually in the United States (Bradford et al. 2009; Wong et al. 2011). The etiology of CTCL is unknown. Although several groups have suggested a potential viral etiology for CTCL, none have been confirmed (Mirvish et al. 2011). Furthermore, case-control studies have not demonstrated any relationship between the environmental, occupational or chemical exposures and the risk of developing CTCL (Whittemore et al. 1989; Morales Suarez-Varela et al. 2000). One study has reported the incidence of CTCL within a family (Sandbank & Katzenellenbogen 1968). However, CTCL is considered to be a sporadic disease. The main representatives of CTCL are the skin-associated Mycosis Fungoides (MF) and its leukemic variant Sezary syndrome (SS), which together account for about 75% of all CTCL cases (Kari et al. 2003; Klemke et al. 2006; Olsen et al. 2007; Bradford et al. 2009). The characteristic of both MF and SS is the monoclonal proliferation of small to medium sized, cytologically atypical
mature CD4+CD45RO+ memory T-cells with cerebriform nuclei (Harris et al. 1994; Diamandidou et al. 1996). CTCL is more common in men than in women (2.2:1 ratio) and those of African-American lineage have a 50% increased risk of developing this disease (Weinstock & Horm 1988; Diamandidou et al. 1996; Criscione & Weinstock 2007).

1.3.1 Mycosis Fungoides

MF accounts for about 72% of all CTCL cases and is considered to be the most common subtype (Hwang et al. 2008). MF was first described as mushroom-shaped skin tumors by French physician, Jean Louis Alibert in 1806 (Makdisi & Friedman 2013). In MF, small or medium sized CD4+CD45RO+ mature memory T-cells with cerebriform nuclei infiltrate the epidermis. The majority of these malignant T-cells also co-express skin-homing molecules such as cutaneous lymphocyte antigen (CLA) and chemokine (C-C motif) receptor 4 (CCR4) (Ferenczi et al. 2002; Campbell et al. 2010). These malignant T-cells usually show the phenotype consistent with effector memory T-cells by losing the expression of the lymph node homing molecules, CCR7 and L-selectin, as well as the lack of expression of CD27 (Campbell et al. 2010). One of the hallmarks of MF is the presence of Pautrier’s microabscesses in epidermis, which are clusters of malignant lymphocytes in close association with Langerhans cells (LCs) (Willemze et al. 2005; Hwang et al. 2008). The presence of this immunophenotypic hallmark raises the hypothesis that the cytokines produced by LCs and the constant stimulation of T-cells by these cytokines are important for clonal expansion of T-cells and their migration into the epidermis (Hwang et al. 2008; Wong et al. 2011). The early manifestations of MF are the development of itchy and dry patches and more infiltrated plaques on the skin, especially in areas rarely exposed to sunlight (Willemze et al. 2005; Hwang et al. 2008). In the early stages,
MF symptoms mimic many benign dermatoses such as eczema and psoriasis and therefore, multiple biopsies are required to reach a definite diagnosis (Hwang et al. 2008). Early-stage MF is an indolent disease, but rapid progression into tumor stage and extracutaneous manifestations can also occur in some patients (Willemze et al. 2005; Hwang et al. 2008). The 5-year survival rate for early-stage MF is about 90%, but this can significantly change by the stage of diagnosis and any evidence of extracutaneous involvement (Willemze et al. 2005). MF incidence increases with age with a median age at diagnosis of 55-60 years; yet, the disease has also been reported in children and adolescents (Willemze et al. 2005). In early-stage MF, the Th1 cytokine pattern has been described, which is characterized by the production of IL-2, IL-12, and IFN-γ. However, as MF progresses to the advanced stage and the number of CD4+ T-cells increases, the Th2 cytokine pattern becomes more dominant with an increase in the expression of IL-4, IL-5, IL-10, and IL-13 (Hwang et al. 2008). It has been shown in a study that MF patients with more CD8+ cytotoxic T-cells in their skin have a better prognosis, possibly due to the immune response and anti-tumor activity of these cells (Hoppe et al. 1995). One of the characteristics of patients with MF, especially at later stages of the disease, is the significant reduction in TCR diversity compared to the greatly diverse TCR repertoires on normal T-cells (Yawalkar et al. 2003). This reduction in TCR diversity results in a profound immunosuppression in late-stage patients with MF to a similar degree as seen in patients with advanced Human Immunodeficiency Virus (HIV), which eventually results in their death due to opportunistic infections (Yawalkar et al. 2003).

The general goal of treatment for CTCL is to control the disease and improve its symptoms while limiting toxic effects to a minimum. In early-stage MF with patches and plaques, skin-targeted therapies such as photochemotherapy, radiotherapy, topical steroids, or
bexarotene gel are considered as first line treatments (Willemze et al. 2005; Wong et al. 2011). Furthermore, biological and immune enhancing therapies such as cytokines and retinoids are also used in the treatment of MF. Chemotherapy is mostly used in cases of systemic involvement and extracutaneous manifestations (Kaye et al. 1989; Willemze et al. 2005; Guenova et al. 2014).

1.3.2 Sezary Syndrome

Compared to MF, SS is a less common but more aggressive subtype of CTCL which was first described by French dermatologist, Albert Sezary, in 1938 (Steffen 2006). Previously, SS had been considered as the advanced stage of MF. However, based on the new classification by the World Health Organization (WHO) and European Organization for Research and Treatment (EORTC), MF and SS are considered as two separate disorders (Willemze et al. 2005). SS occurs exclusively in adults and is characterized by pruritic erythroderma covering over 80% of the body, general lymphadenopathy, and the presence of atypical CD4+CD45RO+ mature memory T-cells with cerebriform nuclei, so-called Sezary cells, in skin, lymph nodes, and peripheral blood (Kari et al. 2003; Willemze et al. 2005; Hwang et al. 2008). Sezary cells often lose the expression of the normal T-cell markers CD7 and CD26 and have a Th2 cytokine profile characterized by the production of IL-4, IL-5 and IL-10 (Kari et al. 2003; Papadavid et al. 2003; Willemze et al. 2005; Hahtola et al. 2006; Hwang et al. 2008). Sezary cells show the phenotype consistent with central memory T-cells by co-expressing the lymph node homing molecules such as CCR7 and L-selectin, as well as the differentiation marker CD27, which is lost upon differentiation into effector memory T-cells (Campbell et al. 2010). The diagnosis criteria for SS, which is recommended by the WHO-EORTC include one or more of the following: CD4/CD8 ratio >10, presence of a T-cell clone in the peripheral blood, an absolute Sezary cell
count of at least 1,000 cells/mm³, CD4⁺CD7⁻ population >40% or CD4⁺CD26⁺ population >30% (Willemze et al. 2005).

In comparison to MF, SS is a very aggressive disease and its 5-year survival rate is only 24% (Willemze, et al. 2005). Similar to the late-stage MF, most patients with SS have a significant reduction in their TCR diversity and they usually die of opportunistic infections due to profound immunosuppression (Yawalkar et al. 2003; Willemze et al. 2005). One of the effective therapy options for SS is the use of Extracorporeal photopheresis (ECP), either alone or in combination with other therapies including interferon-alpha (Willemze et al. 2005; Prince et al. 2009; Booken et al. 2010). During the ECP therapy the patient's blood is temporarily drawn from the body, the WBCs are separated and then treated with photoactivatable 8-methoxypsoralen, which makes them more sensitive to the ultraviolet (UV) light. Afterwards, the WBCs are exposed to the UV light, which can potentially trigger apoptosis. Furthermore, better response rates have been reported for ECP when used in combination with bexarotene, PUVA, and IFN-α (Prince et al. 2009). The overall response rates to ECP in patients with SS ranges from 30% to 80%. The variance is due to different treatment regimens, different combination therapies, patient selection, and intervals between diagnosis and treatment (Prince et al. 2009; Booken et al. 2010). Moreover, histone deacetylase inhibitors, such as vorinostat, are also used in treatment of SS. An overall response rate of 24-30% has been reported for vorinostat among patients with highly refractory CTCL (Gardner et al. 2009). Furthermore, it is recommended that chemotherapeutic drugs, such as gemcitabine, pentostatin, and methotrexate should be used only in younger patients (Prince et al. 2009).
1.3.3 Candidate Genes and miRNAs Involved in CTCL Pathogenesis

Characterization of the pathogenesis and genetic abnormalities of rare diseases is often slow, as disease complexity is difficult to surmount with a small number of patient samples. During the last ten years, several genetic studies have been conducted on primary MF and SS samples in order to achieve one of the following aims: 1) to establish biomarkers for MF and SS diagnosis, 2) to identify genes important to MF or SS pathogenesis, and 3) to discover potential therapeutic targets (Dulmage & Geskin 2013). A number of genes involved in survival pathways, such as NFκB and STAT3, have been shown to be constitutively active in SS (Eriksen et al. 2001; Sors et al. 2006). Conversely, mutations in several tumor suppressors (p53, p16, p15, and PTEN) and the loss of expression of several apoptotic-related genes (Fas and FasL) have been frequently reported in MF and SS (Hwang et al. 2008; Dulmage & Geskin 2013).

Furthermore, overexpression of several genes, many of which are known oncogenes, have been found in both MF and SS primary samples, namely TWIST1, CD52, JUNB, TOX, and PTPRCAP (Dulmage & Geskin 2013). However, several other genes are differentially expressed between MF and SS, such as the tyrosine kinase receptor EPHA4, which is upregulated in SS primary samples and is downregulated in MF skin biopsies (Dulmage & Geskin 2013). It has been demonstrated in a study that a combination of five genes (STAT4, GATA-3, PLS3, CD1D, and TRAIL) can reliably identify patients with SS with 90% accuracy (Nebozhyn et al. 2006). Moreover, the overexpression of CDO1 and DNM3 in SS samples has been shown to be able to distinguish SS from MF and other inflammatory skin disorders, with high sensitivity (Booken et al. 2008). As described in Sections 1.3.1 and 1.3.2, both SS and advanced-stage MF display Th2 cytokine profile. In this context, a set of Th1-specific genes (TBX21, SCYA5, and NKG7) has been shown to be downregulated in both MF and SS, whereas JunB, a transcription factor
involved in Th2 differentiation, was upregulated (Hahtola et al. 2006; Dulmage & Geskin 2013). Moreover, a study on recurrent genetic alterations in SS, using array comparative genomic hybridization, has identified the gain of c-MYC and loss of c-MYC antagonists (MXI1 and MNT) in 75% and 40%-55% of patient samples, respectively (Vermeer et al. 2008). Also, the same study showed that in the majority of patients with SS, the IL-2 pathway is also affected by gain of IL-2 and IL-2 receptor together with a loss of TCF8 and DUSP5, inhibitors of IL-2 signaling and IL-2 production (Vermeer et al. 2008). In addition to these studies, our group has identified the AHI-1 oncogene and subsequently the BIN1 tumor suppressor gene as potential regulators in CTCL pathogenesis (Ringrose et al. 2006; Kennah et al. 2009), which will be further discussed in Sections 1.4 and 1.5, respectively.

In addition to studies focusing on gene expression changes and genetic alterations, several research groups have recently investigated the expression of microRNAs (miRNAs) in both SS and MF. For instance, miR-21 is upregulated in SS and acts as a negative prognostic factor (Narducci et al. 2011; van der Fits et al. 2011). Furthermore, miR-342 and miR-17 with pro-apoptotic activities have been shown to be downregulated in SS (Ballabio et al. 2010). Other recent studies have demonstrated the upregulation of miR-155 in MF, which promotes tumor cell proliferation (van Kester et al. 2011; Kopp et al. 2013). Strikingly, it has been demonstrated that a panel of five miRNAs (miR-326, miR-711, miR-663b, miR-203, and miR-205) can distinguish CTCL from benign skin disorders with more than 90% accuracy (Ralfkiaer et al. 2011). So far, these studies indicate that miRNAs play important roles in CTCL pathogenesis and they may also be useful in CTCL diagnosis. It is evident that there is still much ground to cover to determine the genetic pathogenesis behind SS and MF with the hopes of eventually developing more effective diagnostic tools and targeted therapies for these diseases.
1.3.4 Importance of Apoptosis Resistance in CTCL

It has been reported that the accumulation of malignant T-cells in SS and MF is not due to increased proliferation, but due to defects in the apoptosis pathways, which contribute to apoptotic resistance of CTCL cells (Dummer et al. 1995; Braun et al. 2007; Contassot et al. 2008; Hwang et al. 2008; Wang et al. 2011). Neoplastic T-cells from the early stages of CTCL display a low proliferation rate based on Ki-67 activity, which is a proliferation-associated human nuclear antigen (Dummer et al. 1995). In addition to the low proliferation rate, neoplastic cells in skin lesions of patients with CTCL rarely demonstrate apoptosis as assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate end labeling (TUNEL) assay, which detects DNA fragmentation (Nevala et al. 2001). Moreover, unlike their normal counterparts, CTCL cells are resistant to apoptosis following treatment with most chemotherapeutic drugs, further suggesting the presence of apoptotic defects in these cells (Meech et al. 2001).

Interestingly, one of the major pathways mediating apoptotic activity in T-cells and maintaining T-cell homeostasis is the extrinsic Fas apoptosis pathway (Takahashi et al. 1994; Li et al. 1999). The death-inducing signaling complex (DISC) is formed after the ligation of Fas receptor with FasL on the cell surface. DISC includes the Fas-associated death domain protein (FADD) and pro-caspase 8 that interact with each other through their death effector domains (DED). The autoproteolytic cleavage of caspase 8 in the complex activates downstream effector caspases, such as caspase 3, which results in the cleavage of several proteins called “cell-death substrates” and leads to apoptosis (Figure 1.4) (Nagata 1999; Meech et al. 2001; Krammer et al. 2007). c-FLIP is a critical inhibitor of extrinsic apoptosis pathways, including the Fas-mediated pathway (Thome & Tschopp 2001; Krammer et al. 2007). c-FLIP has multiple splice variants at
the transcript level, while only two major isoforms at the protein level have been described, termed c-FLIP<sub>s</sub> (short) and c-FLIP<sub>L</sub> (long) (Krueger et al. 2001; Thome & Tschopp 2001). These two c-FLIP isoforms are highly homologous to caspase 8 and contain a DED through which they can bind to FADD, block the autoproteolytic processing of caspase 8 and inhibit the cell-death-receptor-induced apoptosis (Thome & Tschopp 2001; Krammer et al. 2007). In particular, defects in the Fas apoptosis pathway are frequently observed in primary MF and SS patient samples as well as in CTCL cell lines (Valente et al. 2006; Braun et al. 2007; Contassot et al. 2008; Wu et al. 2009; Wang et al. 2011; Wu et al. 2011). These defects include altered expression of Fas receptor or its ligand FasL, lack of caspase 8 activation, and c-FLIP overexpression (Braun et al. 2007; Contassot et al. 2008). In addition, the expression of JunB, which negatively regulates the FasL expression, is upregulated in CTCL (Dulmage & Geskin 2013). Nevertheless, the molecular basis for the apoptosis resistance of CTCL cells is not well understood. Identifying critical proteins/pathways involved in the regulation of resistance of apoptosis in CTCL may lead to developing new therapeutic approaches for reversing this resistance.
Figure 1.4: Schematic model of the extrinsic Fas-mediated apoptosis pathway

The death-inducing signaling complex (DISC) is formed after ligation of Fas receptor with Fas ligand on the cell surface. DISC includes Fas-associated death domain protein (FADD) and pro-caspase 8 that interact with each other through their death domains. The autoproteolytic cleavage of caspase 8 in the complex activates the downstream effector caspases, such as caspase 3 which results in the cleavage of several cell-death substrates and leads to apoptosis. c-FLIP is a critical inhibitor of the Fas-mediated pathway, which blocks the autoproteolytic processing of caspase 8 and inhibits the cell-death-receptor-induced apoptosis.
1.4 Abelson Helper Integration Site-1 (AHI-1)

Abelson-murine leukemia virus (A-MuLV) is a replication-defective murine retrovirus containing the v-abl oncogene which is partially responsible for its transforming potential in murine models of leukemia and lymphoma (Abelson & Rabstein 1970; Goff et al. 1980; Goff et al. 1981; Esmailzadeh & Jiang 2011). It is also shown that the expression of v-abl alone is not sufficient to induce full malignant transformation in several mouse strains, suggesting additional genetic events may be required (Green et al. 1987; Savard et al. 1987). A-MuLV is dependent on a non-defective helper virus to be able to replicate in vitro and in vivo, and studies have shown that the helper virus may also be involved in the resulting lymphomagenesis (Poirier et al. 1988). Interestingly, the Abelson helper integration site-1 (Ahi-1) locus was identified in 16% of A-MuLV-induced pre-B lymphomas (Poirier et al. 1988). Later on, the Ahi-1 locus was also identified as a provirus insertional site in other murine leukemia and lymphomas including the c-myc-induced murine T-cell leukemia (Girard et al. 1996), Moloney MuLV-induced rat thymomas (Villeneuve et al. 1993), and acute myeloid leukemias arising in Nf1 heterozygous mice (Blaydes et al. 2001). These findings suggest that the Ahi-1 locus is the target of provirus insertional mutagenesis and its deregulation may contribute to multiple types of murine leukemia and lymphomas. Furthermore, cloning of the Ahi-1 cDNA showed that it encodes a 1047 amino acid protein with a number of interesting domains characteristic of a signaling protein (Jiang et al. 2002).

1.4.1 Structural Analysis and Expression of AHI-1

Human AHI-1 is mapped to a 200 Kilobase (Kb) region of chromosome 6. AHI-1 is highly conserved in mammals and it has at least three isoforms, indicating that the gene
undergoes alternative splicing (Jiang et al. 2002). Furthermore, AHI-1 encodes a unique protein with a Src homology 3 (SH3) domain, multiple SH3 binding sites (PxxP), and multiple WD40-repeats, all known mediators of protein-protein interactions (Figure 1.5) (Jiang et al. 2002; Esmailzadeh & Jiang 2011). AHI-1 also harbors several PEST sequences, known to mediate protein degradation (Rogers et al. 1986) and two potential tyrosine phosphorylation sites. Moreover, the human AHI-1 contains a coiled-coil domain in its N-terminal region, which is also involved in protein-protein interactions (Mason & Arndt 2004). This domain is entirely absent in the mouse and rat Ahi-1 proteins (Jiang et al. 2002; Esmailzadeh & Jiang 2011). Thus AHI-1 has multiple features of a unique adaptor protein regulating specific signaling pathways.

Both mouse and human Ahi-1/AHI-1 are highly expressed in brain and testis and have lower expression in liver, lung, thymus, kidney, and pancreas (Jiang et al. 2002; Doering et al. 2008; Esmailzadeh & Jiang 2011). In the hematopoietic system, in both human and mouse, Ahi-1/AHI-1 is expressed at its highest level in the most primitive HSCs and is rapidly downregulated as cells differentiate. There is an overall 6-fold decrease in human AHI-1 transcript levels from the most primitive lin-CD34+CD38- subset to the most mature lin+CD34- cells in normal adult human BM cells. In addition, within the human lin+ BM cells, the granulocyte lineage shows a significant reduction in the expression of AHI-1 as compared to T, B, and erythroid cell lineages (Jiang et al. 2004; Esmailzadeh & Jiang 2011). The conserved pattern of changes in AHI-1 expression during multi-step hematopoietic cell differentiation suggests that AHI-1 may play important roles in the regulation of the normal hematopoietic stem cell-renewal program and downstream cell differentiation events.
**Figure 1.5: Schematic representation of the mouse Ahi-1 and human AHI-1 proteins**

A summary of the structural motifs present in the Ahi-1/AHI-1 proteins is shown, including: one SH3 domain (red box), seven WD40 repeats (blue triangles), proline-rich motifs (PXXP), PEST sequences (green boxes) and tyrosine kinase phosphorylation sites (Y). The human AHI-1 contains an additional coiled-coil domain which is absent from the mouse Ahi-1 (purple ovals). AHI-1 isoform II, the shortest isoform, lacks the SH3 domain and isoform III, which is still shorter than isoform I, contains additional coding sequences in its C-terminus, which are absent from both isoform I and II.
1.4.2 AHI-1 in Cancer

The first evidence that AHI-1 may be involved in the regulation of human leukemia and lymphoma development is based on an observation that AHI-1 transcripts are significantly higher in a broad spectrum of established human leukemic and lymphoid cell lines compared to the normal human BM. Strikingly, the highest expression levels of AHI-1 are detected in two CTCL cell lines, Hut78, derived from a blood sample of a patient with SS, and Hut102, derived from the blood of a patient with MF, where increases in the AHI-1 transcripts up to 40-fold have been detected compared to the normal BM (Jiang et al. 2004). Furthermore, in primary Sezary cells (CD4+CD7-) from patients with SS, AHI-1 is expressed at significantly higher levels compared to normal CD4+ T-cells from normal controls (Ringrose et al. 2006; Kennah et al. 2009). The direct involvement of AHI-1 in the lymphomagenic activity of Hut78 cells has been shown through stable knockdown of AHI-1 in these cells using retroviral-mediated RNA interference (Ringrose et al. 2006). Knockdown of AHI-1 expression in Hut78 cells reduces autocrine production of IL-2, IL-4, and TNFα; decreases the growth-factor-independence of these cells in vitro; and prevents the tumorigenesis activity of Hut78 cells in immunodeficient mice (Ringrose et al. 2006). Thus, lymphomagenic activity of Hut78 cells is at least partially dependent on the expression of AHI-1. Several differentially expressed genes that may play critical roles in AHI-1-mediated leukemic transformation in CTCL have recently been identified in Hut78 cells with stable AHI-1 suppression through microarray analysis (Kenhah et al. 2009; Esmailzadeh & Jiang 2011). One of these candidates is Bridging integrator 1 (BIN1), which is upregulated at both RNA and protein levels in AHI-1-suppressed cells compared to control Hut78 cells. In addition, BIN1 transcript levels are significantly downregulated in primary CD4+CD7- Sezary cells compared to controls (Figure 1.6) (Kenhah et al. 2009; Esmailzadeh &
Jiang 2011). Identifying the role of BIN1 in CTCL and investigating its potential link to the AHI-1 oncogene are further described in Chapters 3 and 4 of this thesis.

In addition to the role of AHI-1 in CTCL, its deregulated expression has also been demonstrated in human chronic myeloid leukemia (CML). AHI-1 is significantly upregulated in a CML cell line K562 as well as in Philadelphia chromosome positive primary leukemic stem and progenitor cells (Jiang et al. 2004). Overexpression of AHI-1 in primitive hematopoietic cells enhances their growth kinetics in vitro and induces leukemia in vivo (Zhou et al. 2008). AHI-1 can physically interact with BCR-ABL and JAK2, sustain BCR-ABL phosphorylation, and enhance activation of the JAK2/STAT5 pathway in CML cells (Zhou et al. 2008; Esmailzadeh & Jiang 2011). Furthermore, a recent study has demonstrated that disrupting the AHI-1–BCR-ABL–JAK2 interaction complex in CML stem/progenitor cells may improve the response in patients that are resistant to imatinib treatment (Chen et al. 2013). Interestingly, it has been reported that blood samples obtained from CML patients under imatinib therapy have significantly higher levels of AHI-1 than newly diagnosed samples from a total of 38 patients, suggesting that expression changes of AHI-1 may predict response of the patients to tyrosine kinase inhibitors (TKIs), such as imatinib (Balci et al. 2011).
Figure 1.6: Identification of BIN1 as a candidate gene involved in AHI-1-mediated leukemic transformation in CTCL

(A) Venn diagram of differentially expressed genes from the microarray analysis selected by both Limma and dChip analyses. Affymetrix GeneChip microarray analysis identified several differentially expressed genes in AHI-1-suppressed cells compared to Hut78 and empty vector controls. Initial Limma analysis selected 283 differentially expressed probe sets, which was further refined to 33 with the Benjamini and Hochberg P-value adjustment. (B) Protein expression of BIN1 in AHI-1-suppressed cells (sh4-bulk and sh4-clone 1) compared to Hut78 and empty vector (Hut78/RPG) controls. The 65 Kd isoform of BIN1 shows upregulation in the absence of AHI-1. (C) The mRNA expression level of total BIN1 is significantly downregulated in six SS patients (red bars) compared to five CD4+ T cell samples from normal controls (blue bars).
1.4.3 AHI-1 beyond Cancer

In addition to the role of AHI-1 in the development of leukemia and lymphoma, studies have also demonstrated its altered function in brain disorders such as Joubert syndrome (JS) and JS related disorders (JSRD), schizophrenia and autism (Dixon-Salazar et al. 2004; Ferland et al. 2004; Amann-Zalcenstein et al. 2006; Parisi et al. 2006; Ingason et al. 2007; Alvarez Retuerto et al. 2008; Esmailzadeh & Jiang 2011). Genetic pedigree analysis has demonstrated a high linkage and association between the AHI-1 locus and JS, a rare autosomal recessive disorder characterized by abnormal brain development and mental retardation (Dixon-Salazar et al. 2004; Ferland et al. 2004; Esmailzadeh & Jiang 2011). A high frequency of AHI-1 mutations can be identified in patients with JS, with most mutations being frameshift or nonsense mutations which result in a truncated N-terminal AHI-1 or a loss of the WD40-repeat and/or SH3 domains (Ferland et al. 2004; Esmailzadeh & Jiang 2011). Recently, several linkage and association studies have further identified AHI-1 as a susceptibility gene for schizophrenia and autism (Amann-Zalcenstein et al. 2006; Ingason et al. 2007; Alvarez Retuerto et al. 2008; Esmailzadeh & Jiang 2011). These findings suggest an important role for AHI-1 in common brain disorders affecting human cognition and behavior. Furthermore, patients with JSRD can also develop the additional symptoms of retinal degeneration and nephronophthisis (Valente et al. 2003; Gleeson et al. 2004). It has been reported that Ahi-1 knockout mice have normal embryonic development and preserved brain morphology; however, the majority of these mice do not survive to adulthood due to the development of the cystic kidney disease nephronophthisis (Lancaster et al. 2009; Esmailzadeh & Jiang 2011). Moreover, these mice can develop retinal degeneration similar to the retinal phenotype observed in patients with JSRD (Louie et al. 2010; Westfall et al. 2010; Esmailzadeh & Jiang 2011). These findings further highlight the important role of AHI-1
in JS and its related disorders. In conclusion, understanding molecular functions of the AHI-1 gene could provide important insights into processes involved in specific types of diseases and may set the stage for translation into new and more effective diagnostic and/or treatment strategies.

1.5 Bridging Integrator 1 (BIN1)

BIN1 is a nucleocytoplasmic adaptor protein that was first identified through a yeast 2-hybrid screen as one of the potential interacting proteins of the c-MYC oncprotein (Sakamuro et al. 1996). The protein was originally named Box-dependent myc-interacting protein-1. However, structural analysis of BIN1 demonstrated the presence of a SH3 domain, which mediates protein-protein interactions in signaling pathways (Mayer 2001; Rebye et al. 2012). Thus, the potential association to signal transduction led to an alternative name for BIN1, Bridging Integrator-1. BIN1 is one of the archetypal members of the BAR (Bin/Amphipysin/RVS) adapter gene family that are highly conserved through evolution from yeast to human. Members of the BAR adapter proteins are involved in different cellular processes, such as endocytosis, actin organization, and tumor suppression activities (Sakamuro et al. 1996; Prendergast et al. 2009).

1.5.1 The Splicing and Structural Analysis of BIN1

Human BIN1 gene maps to chromosome 2q14.3 and consists of 19 exons (Figure 1.7). BIN1 transcripts are subject to alternative splicing which leads to generation of more than 10 isoforms with diverse patterns of distribution between tissues, sub-cellular localization, and protein interactions. However, most isoforms vary mainly in their inclusion of exons 10, 12A-D,
and 13 (Sakamuro et al. 1996; Wechsler-Reya et al. 1997; Ren et al. 2006; Prendergast et al. 2009). BIN1 contains four important protein domains, termed the N-terminal BAR (exons 1-9), neural-tissue specific (NTS) (exons 12A-D), MYC-binding (MBD) (exons 13-14), and SH3 (exons 15-16) domains (Figure 1.7). Furthermore, exon 10, previously shown to be muscle-specific, encodes a short polybasic sequence which may potentially target BIN1 to membrane compartments such as T-tubules in muscle cells and also has a role in myoblast differentiation (Wechsler-Reya et al. 1998; Lee et al. 2002). The ubiquitously-expressed BAR domain binds to lipid membranes in a dimer conformation and has a role in promoting membrane curvature (Peter et al. 2004; Frost et al. 2009). In addition, this domain is important for the anti-proliferative role of BIN1 in malignant cells (Elliott et al. 1999). The NTS domain, which is found only in central nervous system (CNS) isoforms, can bind to several endocytic proteins and has a role in endocytosis (Butler et al. 1997; Ramjaun & McPherson 1998). The MBD region is important for interaction with the MYC oncogene and inhibiting its transforming activity. Alternative splicing of exon 13 leads to loss of this interaction between MYC and BIN1 (Elliott et al. 1999). The ubiquitously-expressed SH3 domain binds to proline-rich motifs and has been shown to be important in inhibiting the transforming activity of several oncogenes including adenovirus early-region 1A (E1A) (Yu et al. 1994; Elliott et al. 1999).

It has been reported that the CNS isoforms of BIN1 are exclusively cytoplasmic and have roles in endocytosis and membrane trafficking. However, the ubiquitous and muscle-specific isoforms of BIN1 are unique in their ability to localize to the nucleus in addition to the cytoplasm, a characteristic that is essential to manifest tumor suppressor properties related to anti-proliferation, differentiation, and apoptosis (Prendergast et al. 2009; Prokic et al. 2014).
Figure 1.7: Schematic representation of BIN1 protein domains and its isoforms

(A) Full length BIN1 consists of 20 exons and differential splicing has been reported for exons 10, 12A-D, and 13. BAR = BIN1/Amphiphysin/RVS167-related, U = unique, NTS = neural tissue specific, MBD = MYC binding domain, SH3 = Src homology 3. (B) BIN1 has two ubiquitously expressed isoforms. In addition, several tissue-specific isoforms have been previously reported for BIN1, including muscle-specific, as well as brain and neural-specific isoforms. Furthermore, isoforms with inclusion of exon 12A have been identified in several cancer cell lines and are thought to be cancer-specific.
1.5.2 BIN1 Involvement in Cancer

Several studies, have shown that BIN1 expression is reduced or completely lost in several cancer types including melanoma, neuroblastoma, lung, breast, colon, and prostate cancers (Ge et al. 1999; Ge et al. 2000a; DuHadaway et al. 2003; Tajiri et al. 2003; Prokic et al. 2014). Methylation of the BIN1 promoter has also been reported in fractions of primary breast and prostate cancer samples (Kuznetsova et al. 2007). Furthermore, BIN1 attenuation or mis-splicing correlates with cancer prognosis, increased metastasis and reduced survival, suggesting tumor suppressor activities of BIN1 (Ge et al. 1999; Ge et al. 2000a; Ge et al. 2000b; Ghaneie et al. 2007).

*In vitro* studies have demonstrated that nuclear-localizing isoforms of BIN1 have anti-proliferative and pro-apoptotic roles in solid tumor cell line models and can inhibit the transforming activity of several oncogenes including MYC, adenovirus E1A, and mutant p53. However, the mechanistic bases underlying these effects are not fully understood (Wechsler-Reya et al. 1998; Elliott et al. 1999; Prendergast et al. 2009; Prokic et al. 2014). The overexpression of the nuclear-localizing isoforms of BIN1 reduces the cell growth and colony formation in several cancer cell lines (Sakamuro et al. 1996; Elliott et al. 1999; Hogarty et al. 2000; Tajiri et al. 2003). Moreover, most studies on solid tumor cell line models including hepatocarcinoma, neuroblastoma, and prostate cancer have proposed that BIN1 is involved in a caspase-independent apoptosis process (Elliott et al. 2000; Hogarty et al. 2000; Cassimere et al. 2009). However, one study has demonstrated the effect of Bin1 on caspase-dependent apoptosis in transformed primary mouse embryo fibroblasts (MEFs) (Muller et al. 2004). This study indicated that cleaved caspases and effector events including cleavage of PARP are activated.
only in the BIN1+/+ MEF cells, suggesting a potential role for Bin1 in caspase-dependent apoptosis pathway (Muller et al. 2004).

In contrast to the nuclear-localizing isoforms with tumor suppressor functions, it has been reported that inclusion of exon 12A alone, in the absence of the other 3 neuron-specific exons (12B-D), yields the so-called cancer-specific isoforms, which are observed in several primary tumor cells and tumor cell lines (Figure 1.7). These +12A isoforms usually lack the anti-proliferative and pro-apoptotic roles and cannot inhibit the MYC transforming activity (Galderisi et al. 1999; Ge et al. 1999; Elliott et al. 2000; Pineda-Lucena et al. 2005). However, the BIN1 (+12A) isoforms in human neuroblastoma cell lines have anti-proliferative roles and significantly decrease the numbers of colony output (Tajiri et al. 2003), suggesting that these isoforms may have tissue-specific functions and their biological effects on cell proliferation or apoptosis may change in specific tissues and cell types from different malignancies.

Several studies have investigated the biological effects of Bin1 suppression in primary transformed MEFs, as well as in mouse models. Targeted deletion of Bin1 in c-myc/H-ras-transformed MEFs results in increased cell proliferation in vitro as well as in producing larger tumors in vivo (Muller et al. 2004). Furthermore, it has been shown that mammary gland-specific deletion of Bin1 in mouse is not sufficient for the initiation of breast cancer. However, when breast cancer is initiated by treatment with a carcinogen, Bin1 loss leads to formation of poorly differentiated and more aggressive tumors (Chang et al. 2007b). In addition, in old mosaic Bin1-null mice (18-20 months of age), Bin1 loss is associated with an increased incidence of several cancers such as lung, prostate, colon, and liver cancers as compared to the age-matched controls (Chang et al. 2007a). These studies further propose that Bin1 acts as a negative modifier of oncogenicity and cancer susceptibility in mouse models.
Several other lines of evidence further suggest that BIN1 acts as a negative modifier of oncogenicity. Bin1 loss can promote immune escape of cancer cells by deregulating the immunomodulatory enzyme indoleamine 2, 3-dioxygenase (IDO) that is highly elevated in several human cancer types (Uyttenhove et al. 2003; Muller et al. 2005). Moreover, studies suggest that BIN1 has an important role in DNA repair through binding to and inhibiting the activation of the PARP1 enzyme, which is an essential component of the base excision repair pathway. Thus, a decrease in BIN1 expression reduces sensitivity of malignant cells to the DNA-damaging chemotherapeutic agents such as cisplatin, doxorubicin, and etoposide (Pyndiah et al. 2011; Tanida et al. 2012).

1.5.2.1 Connecting BIN1 to CTCL

As described in Section 1.4.2., our lab has identified BIN1 through a microarray analysis as one of the genes that may be involved in AHI-1-mediated leukemic transformation and may have a role in CTCL pathogenesis (Kennah et al. 2009). It has been demonstrated that BIN1 expression is upregulated at both the RNA and protein levels in the absence of the AHI-1 oncogene in Hut78 cells. Furthermore, transcript levels of total BIN1 have been shown to be significantly lower in primary CD4+CD7− Sezary cells from patients with SS as compared to controls (Figure 1.6) (Kennah et al. 2009). In addition, by conducting exon-specific reverse transcription polymerase chain reaction (RT-PCR), four isoforms of BIN1 have been identified in Hut78 cells, as well as in primary CD4+CD7− Sezary cells from patients with SS, and in CD4+ T-cells from normal controls (Figure 1.8) (Kennah et al. 2009). These four isoforms, based on the presence or absence of exons 10, 12A-D, and 13 are termed as: BIN1 (+10, +12A, +13), BIN1 (+10, +12A, -13), BIN1 (+10, +13), and BIN1 (+10, -13). Similar to total BIN1
expression, the transcript levels of BIN1 (+12A) isoforms are also downregulated in the absence of the AHI-1 oncogene in Hut78 cells (Figure 1.8F). Interestingly, exons 10 and 12A, previously reported to be muscle-specific and cancer-specific, respectively, have been observed in both normal T-cells and Sezary cells (Kannah et al. 2009). These results suggest that exons 10 and 12A are not exclusively specific to muscle differentiation and cancer development and may have additional functions in normal T-cells and T-cell malignancies, such as CTCL.
Adapted from Blood 7, 4646-55, 2009 (with permission)
**Figure 1.8: Characterization of the BIN1 isoforms in CTCL cells using exon-specific RT-PCR**

(A) Schematic representation of protein domains and exon organization of BIN1. The specific primer sets used to detect exons 10, 12 A-D, and 13 are indicated as red arrows: BIN1 9/10 and BIN19/11 primer sets were generated to detect the presence of exon 10 and the BIN1 11/14 set was used to detect the presence of exon 13 and 12A-D. (B) Presence of only one single product from each primer set confirmed the presence of exon 10 in all isoforms by qRT-PCR. Lane 1: Hut78, Lane 2: Hut78 RPG, Lane 3: Hut78/sh4 bulk, and Lane 4: Hut78/sh4 clone 1. (C) Four distinct RT-PCR products were identified and cloned in Hu78 cells using BIN1 11/14 primers. Lane 1: Hut78, Lane 2: Hut78 RPG, Lane 3: Hut78/sh4 bulk, Lane 4: Hut78/sh4 clone 1, Lane 5: negative control (no RNA), Lane 6: positive control (Hut78 RPG with GAPDH primer set). (D) RT-PCR analysis revealed that the four BIN1 isoforms identified in Hut78 and Hut78-transduced cells were also present in both CD4⁺CD7⁻ Sezary cells from SS patients, as well as in normal CD4⁺ T-cell from healthy donors. (E) Schematic diagram of the exon organization of the four BIN1 isoforms characterized in the Hut78 cell lines and primary samples. (F) qRT-PCR analysis using the BIN1 9/10 and BIN1 12A primer sets demonstrated upregulation of total BIN1 and BIN1 (+12A) transcripts in AHI-1–suppressed cells compared to controls.
1.5.3 BIN1 beyond Cancer

Although ubiquitously expressed, the highest expression of BIN1 is found in brain and muscle (Wechsler-Reya et al. 1997). It has been shown that several mutations or mis-splicing in the BIN1 gene can cause different muscle and brain disorders (Claeys et al. 2010; Fugier et al. 2011; Bohm et al. 2013). For example, germline mutations in BIN1 have been documented in autosomal recessive centronuclear myopathy (CNM), a rare disease associated with progressive muscle weakness. To date, several homozygous mutations have been reported in BIN1 which lead to the development of CNM; missense mutations in the BAR domain, stop codon mutations in the SH3 domain and a mutation in exon 10 which causes exon skipping (Nicot et al. 2007; Claeys et al. 2010; Bohm et al. 2013). Furthermore, aberrant splicing of BIN1 can also lead to another type of myopathy, called myotonic muscular dystrophy (MMD) (Fugier et al. 2011). Both CNM and MMD share several features including muscle weakness and increased centralized myonuclei, suggesting potential common molecular mechanisms for BIN1 in these diseases. Moreover, BIN1 is also linked to heart and cardiac failure in both human and mouse. BIN1 is important in cardiomyocyte homeostasis and reduction in plasma BIN1 levels has been documented in patients with ventricular arrhythmia, a disorder involving diseased cardiac muscle (Hong et al. 2012). Also, complete Bin1 knockout causes perinatal lethality in mice due to cardiomyopathy (Muller et al. 2003).

In addition to involvement of BIN1 in different muscle disorders, several large scale genome-wide association studies linked the BIN1 gene locus to late-onset Alzheimer’s disease (AD) (Seshadri et al. 2010; Naj et al. 2011; Kamboh et al. 2012; Tan et al. 2013). AD is a complex neurodegenerative disorder and is the leading cause of dementia in population older than 65 years of age. In fact, BIN1 is the most important genetic risk locus for late-onset AD.
after apolipoprotein E (APOE) (Tan et al. 2013). However, the mechanism of action of BIN1 in AD progression is still not fully understood and further investigation of its molecular and biological functions will be of great value. Altogether, these studies have revealed the importance of BIN1 in human disorders. However, the molecular and cellular bases underlying such different diseases related to alterations in BIN1 are still unclear and represent an exciting area of research.

1.6 Experimental Outline

As described above: (1) BIN1 has been identified by microarray analysis of CTCL cells as a candidate gene involved in AHI-1-mediated lymphomagenesis; (2) Total BIN1 expression is significantly downregulated in primary CD4+CD7- Sezary cells from patients with SS compared to healthy controls (Figure 1.6C); (3) Four BIN1 isoforms have been identified in primary CD4+CD7- Sezary cells and in CTCL Hut78 cells, including BIN1 (+12A) isoforms (Figure 1.8); (4) The expression of total BIN1 and BIN1 (+12A) isoforms is upregulated in the absence of the AHI-1 oncogene in Hut78 cells, suggesting a potential link between AHI-1 and BIN1 (Figure 1.8F); (5) Although BIN1 (+12A) isoforms have been reported to be “cancer-specific” in solid tumors, the presence of exon 12A is found in normal CD4+ T-cells from healthy donors (Figure 1.8D) (Kennah et al. 2009), suggesting that exon 12A is not specific to cancer and may have other functions in human T-cells. Thus, the significant reduction in BIN1 transcripts in SS patients and the upregulation of BIN1 isoforms in the absence of the AHI-1 oncogene in CTCL cells strongly suggest that aberrant expression/activity of BIN1 and its isoforms may alter their tumor suppressor properties, contributing to the pathogenesis of human CTCL. Based on these significant findings, the major goal of this thesis project is to investigate
the role of BIN1 isoforms in the regulation of proliferation, apoptosis and tumor formation of CTCL cells both *in vitro* and *in vivo*, using a lentiviral-mediated gene transduction model system. To our knowledge, this is the first study investigating the biological and molecular effects of BIN1 and its isoforms in a T-cell malignancy.

In Chapter 3 of this thesis work, I have mainly focused on investigating the role of BIN1 and its isoforms in CTCL, using two cell line models: Hut78 cells, derived from the peripheral blood (PB) of a patient with SS, and HH cells, derived from the PB of a patient with aggressive type of CTCL. Hut78 cells express endogenous BIN1, whereas HH cells have no endogenous expression of BIN1. Two key isoforms of BIN1 have been selected for overexpression/restoration and knockdown studies in these two models. One selected isoform is BIN1 (+10, +13), which is the ubiquitous isoform of BIN1 and has been shown to have tumor suppressor properties, with anti-proliferation and pro-apoptotic roles (Sakamuro *et al.* 1996; Prendergast *et al.* 2009). The other isoform is BIN1 (+10, +12A, +13), which only differs from BIN1 (+10, +13) by the inclusion of exon 12A. There are controversial results regarding its anti-proliferative role in solid tumors (see Section 1.5.2.). The molecular and cellular effects of these two isoforms on cell proliferation, apoptosis, and tumor formation have been extensively investigated using *in vitro* and *in vivo* assays.

Furthermore, in Chapter 4, I have explored potential molecular mechanisms by which AHI-1 leads to downregulation of BIN1 by determining physical interactions between BIN1 and AHI-1 or other proteins in BIN1-transduced cells using co-immunoprecipitation (IP)/mass spectrometry analysis and co-localization studies. I have also examined whether AHI-1 regulates transcription of BIN1 through epigenetic changes by studying the methylation status of the BIN1 promoter using bisulfite sequencing.
1.6.1 Hypotheses

- BIN1 and its isoforms act as tumor suppressors in the regulation of cell proliferation and specific apoptosis pathways in human CTCL.
- AHI-1 regulates the expression of BIN1 and causes the latter to lose its tumor suppressor activity, thereby mediating the cell proliferation and apoptosis in human CTCL.

1.6.2 Objectives

- To investigate the biological and molecular functions of BIN1 isoforms, both in vitro and in vivo, by lentiviral transduction of BIN1 (+10, +13) and BIN1 (+10, +12A, +13) isoforms into human CTCL cells and assessing the effects.
- To investigate the potential molecular mechanisms by which AHI-1 leads to downregulation of BIN1 and to identify additional BIN1 interacting proteins in BIN1-transduced CTCL cells by co-IP/mass spectrometry analysis, co-localization studies and epigenetic analysis of the BIN1 promoter region.

1.6.3 Specific Aims

Chapter 3:

1. To establish new cell line model systems by constructing lentiviral vectors containing the two key BIN1 isoforms and stably transducing them into CTCL cells (Hut78 and HH cells).
2. To investigate the sub-cellular localization of the BIN1 isoforms in CTCL cells.
3. To investigate the biological effects of BIN1 overexpression/restoration in the regulation of cell proliferation and specific apoptosis pathways in CTCL cells.
4. To investigate the biological effects of BIN1 overexpression/restoration on tumor formation using the immunodeficient NOD/SCID mouse model.

Chapter 4:

1. To investigate the potential protein-protein interactions between AHI-1 and BIN1 in BIN1-transduced CTCL cells using co-IP/mass spectrometry.

2. To identify the potential BIN1-interacting proteins in BIN1-transduced CTCL cells by mass spectrometry and co-localization studies.

3. To investigate the sub-cellular localization of AHI-1 in CTCL cells.

4. To investigate the potential effects of AHI-1 on methylation status of the BIN1 promoter using bisulfite sequencing.
Chapter 2: Materials and Methods

2.1 Human Primary Samples

Skin biopsies from MF patients at diagnosis (n = 15) and controls with benign inflammatory dermatoses (atopic dermatitis, n = 9) were obtained from the Department of Dermatology, University of British Columbia (UBC, Appendix Table A.1). Full-thickness lesional skins were obtained by 4-mm punch biopsies under local anesthesia. Skin biopsies were immediately placed in RNAlater and used for RNA extraction by TRIZOL reagent (Invitrogen, Burlington, ON, Canada). Peripheral blood (PB) from 13 SS patients at diagnosis, 6 benign inflammatory dermatoses (vitiligo = 3, psoriasis = 3) and 5 normal individuals was obtained from the Department of Dermatology, UBC (Appendix Table A.2). Informed consent was obtained in accordance with the Declaration of Helsinki, and the procedures used were approved by the Research Ethics Board of the University of British Columbia.

All patients were screened for human T-cell leukemia virus-1 status and were negative. Leukemic Sezary cells and normal blood cells were purified by negative selection with monoclonal antibodies directed against granulocytes, B cells, and CD8+ and CD7+ T cells using a RosetteSep kit (STEMCELL Technologies, Vancouver, BC, Canada) as previously described (Kennah et al. 2009). Cell purity was verified by fluorescence-activated cell sorter (FACS), with more than 90% purity by immunophenotyping (CD4+CD7−) in the majority of samples, as described (Kennah et al. 2009).
2.2 Cell lines and Cell Culture

Human CTCL cell lines, Hut78 and HH (ATCC no. TIB-161 and CRL-2105), were cultured in Rosewell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 0.1 mg/mL streptomycin, and $10^{-4}$ M β-mercaptoethanol (StemCell Technologies, Vancouver, BC), at 37°C, 5% CO2, in a humidified cell culture incubator. These cells were maintained at a cell density less than $1 \times 10^6$ cells/mL as suspension cultures in 10 cm Falcon® tissue culture dishes (Corning Inc., Lowell, MA).

2.3 Cloning and Constructing Lentiviral Vectors

Two BIN1 isoforms, the BIN1 (+10, +13) and BIN1 (+10, +12A, +13) cDNAs in pcDNA3 vector (kindly provided by Dr. G. C. Prendergast, Lankenau Institute for Medical Research, Philadelphia, U.S) were restriction enzyme digested with EcoR1 (Invitrogen). DNA inserts were gel purified and cloned into the pMN DU3-PGK-eGFP (MPG) or pMN DU3-PGK-eYFP (MPY) lentiviral vectors (kindly provided by Dr. D. B. Kohn, UCLA, Los Angeles, CA, USA) through the EcoRI site (Figure 2.1). The Rapid DNA Dephos and Ligation Kit (Roche) was used according to manufacturer’s instructions for dephosphorylation of MPG and MPY lentiviral vectors and ligation of the BIN1 cDNAs into the vectors. This was followed by transformation of the ligation products into the One Shot® MAX Efficiency® DH5α™-T1R Competent Cells (Invitrogen), which were plated on 100 μg/mL ampicillin LB selection plates. PCR was conducted to select positive colonies with the correct DNA insert, which were then expanded in liquid cultures and the plasmids were purified with the GeneJET™ Plasmid Miniprep Kit (Fermentas, Burlington, ON). Restriction enzyme digestion was further used to
verify the plasmids, and confirmed by DNA sequencing using specific forward and reverse primers (McGill University and Genome Québec Innovation Centre).

To generate lentiviral vector for human c-FLIP, the full-length c-FLIP cDNA (Thermo scientific) was sub-cloned into the MPG lentiviral vector between Ascl and PacI sites (Figure 2.1) and confirmed by DNA sequencing (the NAPS Unit, Michael Smith Laboratories, University of British Columbia), using the same cloning procedure as described above.

Figure 2.1: Schematic representation of the pMNDU3-PGK-eGFP lentiviral vector
The pMNDU3-PGK-eGFP (MPG) vector is a HIV-based and self-inactivating (SIN) lentiviral vector. One of its unique features is the replacement of the HIV U3 region of the 5’ LTR with the promoter and enhancer region from the U3 region of the human cytomegalovirus (CMV). An internal promoter (MND), modified from the U3 region of the myeloproliferative sarcoma virus (MPSV), drives transgene expression. A separate internal PGK promoter drives the expression of enhanced GFP (eGFP). MPG contains other regions including a packaging signal (Ψ), a minimal GAG region (ΔGAG), a Rev responsive element (RRE) and a cPPT to increase transduction efficiency.
2.4 Lentivirus Production and Generation of Stably Transduced Cells

Lentiviral vectors were produced by standard calcium phosphate transfection of 293T cells. 24 hours before transfection, a total of $6 \times 10^6$ cells were plated in each 10-cm Falcon® tissue culture dish in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and 100 U/mL penicillin at 37°C, 5% CO2, in a humidified cell culture incubator. The culture medium was changed 2 hours prior to transfection. A total of 10 μg of lentiviral vector DNA, two packaging constructs (6.5 μg of ΔR and 2.5 μg of REV), and 3.5 μg of vesicular stomatitis virus glycoprotein (VSV-G) envelope construct were used for the transfection of each dish. Packaging and envelope plasmids were gifts from P. Leboulch (Harvard University, Boston, MA, USA). Viral supernatant was collected after 24 and 48 hours post-transfection and filtered with a 0.45 μm low protein binding filter before concentration by ultracentrifugation for 90 minutes at 100,000 g. Multiple aliquots of the concentrated virus were stored at -80°C. Viral titers were measured by transfection of HeLa cells with serial dilutions of concentrated virus, and only those lentiviral vectors with a titer of $\geq 2 \times 10^8$ U/mL were used for transducing CTCL cell lines.

To transduce Hut78 and HH cells with lentiviral vectors, $2 \times 10^5$ cells were plated into 1 ml of complete RPMI 1640 media in a 24-well plate. Subsequently, 5 μL of the concentrated virus and 5 μg/mL of protamine sulfate were added to these cells. The cells were incubated at 37°C, 5% CO2, in a humidified cell culture incubator for 24 hours. After 24 hours, transduced cells were washed in Dulbecco’s Phosphate Buffered Saline (PBS) (StemCell Technologies, Vancouver, BC) and re-suspended in complete RPMI 1640 and further expanded in a 37°C incubator for another 4-5 days. Fluorescein-activated cell sorter (FACS) sorting was then performed to purify either single positive (GFP+ or YFP+) or double positive (GFP+/YFP+) cells from transduced cells.
2.5 Transfection of BIN1 siRNA in CTCL Cells

For transient knockdown of BIN1, two FlexiTube GeneSolution BIN1 small interfering RNAs (siRNAs) that target all isoforms of BIN1 (SI00312235 and SI03190964; Qiagen) and scrambled siRNA control (Qiagen) were transfected in $2 \times 10^5$ cells with 400 nM of BIN1 siRNAs or scrambled siRNA control, using the HiPerFect transfection reagent (Qiagen), according to manufacturer’s instructions. Cells were then recovered after 48 hours before conducting further experiments.

2.6 Total Cell Lysate Extraction and Quantification

Total cell lysate was prepared by pelleting cells after washing with PBS (StemCell Technologies, Vancouver, BC) and placing them for 10 minutes at -80°C to freeze dry. Later, the protein was extracted by re-suspending the pellet in lysis buffer (~25 μL lysis buffer for $1 \times 10^6$ cells) and the mixture was incubated on a rotator at 4°C for about 1 hour. The lysis buffer consisted of 1 mL phosphorylation solubilization buffer (PSB), 50 μL of NP-40 Alternative Protein Grade Detergent (Calbiochem, Gibbstown, NJ), 5 μL phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, Oakville, ON), and 5 μL protease inhibitor cocktail (PIC) (Sigma-Aldrich, Oakville, ON). The total protein lysate was then separated by centrifugation at 12,000 rpm for 10 minutes at 4°C, and the supernatant was then collected and stored at -80°C. The Bradford assay was used to quantify the protein concentration. Bio Rad Protein Assay Dye Reagent (Mississauga, ON) was diluted in deionized super Q water at a ratio of 1:4 times. Protein lysates were diluted at a ratio of 1:10 by adding 5μL of the protein lysate to 45 μL of PSB. Purified bovine serum albumin (BSA, Bio-Rad Laboratories, Mississauga, ON) was used to generate a standard curve, using concentrations ranging from 33.3-1000 ng/μL. 20 μL of
diluted protein and 200 μL of Bio-Rad dye were added to each well of a 96-well Falcon plate and samples were incubated at room temperature (RT) for 10 minutes. The absorbance of standards and samples was measured at 630 nm using the Elx808™ Absorbance Microplate Reader (BioTekk Instruments, Winooski, VT).

2.7 Sub-cellular Fractionation

Transduced-HH cells (~ 5 × 10⁶ cells) were washed twice with ice-cold PBS and re-suspended into 500 μL of hypotonic buffer (10 mM Tris buffer, pH 7.9, containing 3 mM MgCl₂, 10 mM NaCl, 2.5 μL PIC). Re-suspended cells were incubated on ice for 5 minutes and then pelleted in hypotonic buffer by centrifugation at 3,000 × g for 5 minutes at 4°C. The supernatant (cytosolic extract) was transferred into a new tube and the pellet was re-suspended into 100 μL of 10% NP-40 lysis buffer. Re-suspended cells were incubated on ice for 20 minutes and then pelleted in lysis buffer by centrifugation at 13,000 × g for 5 minutes at 4°C. The supernatant (nuclear extract) was transferred into a new tube. Protein concentration for each sub-cellular fraction was measured as described in Section 2.6.

2.8 Western Blotting

Protein expression was assessed by western blotting. Samples were prepared using 30 μg protein lysate, 5 μL loading buffer (4×) and deionized super Q water for a final volume of 20 μL and were heated at 90°C for 10 minutes. Next, samples were separated on a 10%, 12%, or 15% SDS-page gel 1.5 mm, 10 well (Bio-Rad) along with the PageRuler Prestained Protein Ladder (Fermentas, Burlington, ON). The gel was run under reducing conditions at 80 V for 30 minutes and then at 150 V for an hour. Proteins were then transferred from the gel onto Immobilon-P
polyvinylidene fluoride 0.45-μm membrane (Millipore, Billerica, MA) using NuPAGE Transfer Buffer (Invitrogen) at 33 V for 1.5 hours. The membrane was then blocked in Tris-buffered saline Tween 20 (TBST) with 5% skim milk for 1 hour at RT, and washed with TBST for 5 minutes (2 times). The membrane was next incubated with a primary antibody overnight at 4°C, washed with TBST for 15 minutes (3 times), and incubated with horseradish peroxidase–conjugated secondary antibody for 1 hour at RT, followed by 3 × 15-minute washes with TBST. Lastly, target proteins were visualized using enhanced chemiluminescence reagent for 1 minute and then exposed on KODAK BioMax XAR autoradiography film. Conditions used for different primary and secondary antibodies are listed in Table 2.1.

### 2.9 Immunoprecipitation and Mass Spectrometry

Cells were lysed in protein lysis buffer, and protein concentration was determined as described in Section 2.6. For immunoprecipitation (IP), 800 μg of total cell lysate was incubated with 2 μg of the anti-BIN1 mouse monoclonal antibody (Abnova) on a rotator at 4°C overnight. The same amount of normal mouse immunoglobulin G (IgG) (2 μg, Santa Cruz) was also used as a negative control. The next day, the immune complexes were captured by adding 50 μL of protein G bead flurry (Santa Cruz) to the mixture and incubating for another 2 hours on a rotator at 4°C. The beads were then pelleted by spinning the mixture at 14,000 g for 15 seconds and the supernatant was removed and washed with ice-cold PBS (3 times). The samples were then heated at 70°C for 10 minutes and were used for western blotting (see Section 2.8.). The membrane was probed with the anti-human AHI-1 N-terminal rabbit polyclonal antibody (costume antibody production, Applied Biological Materials Inc.) to detect any potential interaction between AHI-1 and BIN1.
To identify the potential BIN1 interacting proteins by mass spectrometry, IP experiments were performed by incubating 1.1 mg of total cell lysate with 2 µg of the BIN1 antibody, as described above in detail. The normal mouse IgG, as well as the beads-only control were used as negative controls to exclude any non-specific interactions. The samples were run on a 10% SDS-PAGE gel. The gel was then stained and visualized with Coomassie® Blue stain on a shaker for 5 minutes at RT followed by de-staining with deionized super Q water overnight. The prominent bands and their cognate slices from control lanes were cut out and processed for tandem mass spectrometry analysis in Dr. Gregg Morin’s laboratory at the Michael Smith Genome Sciences Centre, BC Cancer Agency. Automated in-gel dehydration, alkylation, trypsin digestion, and extraction were performed and samples were analyzed on a 4000 QTrap mass spectrometer (Applied Biosystems/Sciex, Foster City, CA, USA), using standard protocols (Mead et al. 2010). The MS/MS spectra were searched against the Ensembl human database using the Mascot (Matrix Science, Boston, MA, USA) search engine. The candidate interacting proteins were selected based on the identification of at least two unique peptides for each protein and a Mascot score \( \geq 50 \). In addition, the candidate interacting proteins were not found in control samples. The Mascot protein score is representative of both the number of identified unique peptides and the confidence in their identification (Dunham et al. 2012).

2.10 DNA Methylation Analysis of the BIN1 Promoter Region

To investigate the effect of AHI-1 on methylation status of BIN1, bisulfite sequencing of the CpG island of BIN1 promoter region was performed in control and transduced cells. Genomic DNA was extracted from these cells using the AllPrep DNA/RNA Mini Kit (Qiagen). Afterwards, bisulfite conversion of genomic DNA was performed using the EZ DNA
methylation kit (Zymo Research), according to the manufacturer's instructions. Subsequently, nested PCR was performed on bisulfite-treated genomic DNA for amplification of the CpG island of the BIN1 promoter region. The primers used in nested PCR are listed in Table 2.2.

The first PCR (PCR1) was performed under the following thermal profile: 95°C for 10 minutes, followed by 30 cycles of 95°C for 30 seconds, annealing temperature of 50°C for 1 minute, and 72°C for 1 minute. A final elongation step of 72°C for 7 minutes was included in all reactions.

A second PCR (PCR2) was then performed to obtain enough PCR products for further cloning and sequencing analysis. PCR2 was performed under the same thermal conditions as PCR1 and 1 μl of the PCR1 product was used as the template. The PCR products were analyzed by gel electrophoresis and purified with MinElute PCR Purification Kit (Qiagen). The extracted PCR products were then ligated into the pCR®II-Topo® vector using the TOPO TA Cloning® Kit (Invitrogen). This was followed by transformation of the ligation products into the One Shot® MAX Efficiency® DH5α™-T1R Competent Cells (Invitrogen), which were then plated on 100 μg/mL ampicillin LB selection plates with 40 μL of 20 μg/μL X-gal. Positive colonies were then selected by performing PCR and were then sent for sequencing to the McGill University and Genome Québec Innovation Centre.

2.11 RNA Extraction

Total RNA was isolated using the absolutely RNA microprep kit (Stratagene, La Jolla, CA, USA). Briefly, 1 × 10^5 to 1 × 10^6 cells were pelleted and washed in PBS (StemCell Technologies, Vancouver, BC). These cells were then re-suspended in lysis buffer with β-mercaptoethanol. RNA purification was performed using the procedure described for tissue culture cells grown in suspension. Total RNA was then eluted in 30 μL of elution buffer and the
concentration was measured using the nanodrop ND-100 spectrophotometer and measuring optical density at 260 nm and 280 nm.

2.12 Quantitative Reverse Transcription-polymerase Chain Reaction

RNA (0.1-0.5 µg) was reverse transcribed in a 25-µl reaction using 3 µg/µL random primers (Invitrogen), following the protocol for the first-strand cDNA synthesis provided with the SuperScript™ III Reverse Transcriptase (Invitrogen). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using 12.5 µl Power SYBR® Green PCR Master Mix (Applied Biosystems), 1 µl cDNA, 1 µl of the 20-µM gene specific primer (Invitrogen), and 10.5 µl water, for a total reaction volume of 25 µl. Quantification of gene expression was performed using the 7500 Real Time PCR System (Applied Biosystems). The thermal profile was: 50°C for 2 minutes, 95°C for 10 minutes, and 45 or 50 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Fluorescence was calculated using SYBR® as the reporter dye and ROX as the passive reference. Prior to using each primer set, efficiency analysis was performed using serial dilutions of cDNA (1x, 1/10x, 1/100x, and 1/1000x). In all experiments, glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) was used as an endogenous control and data analysis was conducted using the 7500 Real Time PCR System Software (Applied Biosystems). Primers used in this study are listed in Table 2.3.

2.13 Colony-forming Cell Assay

Colony-forming cell (CFC) assays were performed on HH cells using the fetal calf serum-containing methylcellulose (H4230; StemCell Technologies) and on Hut78 cells using the serum-free methylcellulose cultures (H4236, StemCell Technologies). In general, 80 mL of
semi-solid methylcellulose was mixed with 20 mL of Iscove’s Media (Stem Cell Technologies, Vancouver, B.C) and divided into 3-mL aliquots. 600 cells were added to each aliquot and samples were then vortexed, and allowed to incubate at RT for 5 minutes. 3-mL syringes with blunt end needles were then used to plate 1.2 mL of the mixture into CFC plates in duplicate, based on the manufacturer’s instructions (Stem Cell Technologies, Vancouver, and B.C). Colony counts were performed, using standard scoring criteria, on the basis of the ability to produce colonies containing a minimum of 50 cells after 12 to 14 days of incubation at 37°C, 5% CO2, in a humidified cell culture incubator.

2.14 [³H]-thymidine Incorporation Assay

2 × 10⁴ cells were cultured in triplicate in round-bottom wells of 96-well Falcon plates in 100 μl of RPMI 1640 media per well. The cells were cultured for 24, 48, 72, and 96 hours at 37°C, 5% CO2 in a humidified cell culture incubator. The amount of tritiated [³H]-thymidine incorporation during a 4-hour pulse of the culture was measured. Briefly, 1 microcurie (μCi) of tritiated [³H]-thymidine was added to each well. After 4 hours, the cells were harvested onto a membrane using a Skatron instruments combi Harvester (LKB Wallace-PerkinElmer). The amount of tritiated [³H]-thymidine was measured with a LKB Betaplate scintillation counter.

2.15 Apoptosis Assays

To detect spontaneous, as well as the FasL-induced apoptosis, the Annexin V-APC apoptosis detection kit (eBioscience) was used. Briefly, CTCL cells were seeded at 2.5 × 10⁴/mL in RPMI 1640 medium and cultured for 24, 48, or 72 hours at 37 °C, 5% CO2, in a humidified cell culture incubator. For detecting spontaneous apoptosis, HH cells were cultured
in serum-containing RPMI 1640 medium, whereas Hut78 cells were cultured in a more stringent condition, using the serum-free RPMI 1640 medium. After culturing cells for specific time points, cells were pelleted and washed in PBS and re-suspended in 50 μL of 1× Annexin buffer (eBioscience). Subsequently, 2.5 μL of Annexin V-APC (eBioscience), as well as 2.5 μL of propidium iodide (PI) (eBioscience) were added to the cell mixture and incubated for 15 minutes at RT in the dark. After 15-minute incubation, 200 μL of 1× Annexin buffer was added to the mixture and cells were analyzed using a FACSCalibur™ flow cytometer (BD Bioscience) in the Flow Cytometry Core of the Terry Fox Laboratory at the BC Cancer Research Centre. Total apoptotic cell numbers were calculated as the sum of the “early” apoptotic cells (Annexin V+/PI-) and “late” apoptotic cells (Annexin V+/PI+). For the FasL-induced apoptosis, cells were treated with Super FasL (40 ng/mL; Sigma-Aldrich) and incubated for 18 hours before the apoptosis analysis. Data were analyzed with FlowJo software. Specific FasL-induced apoptosis was calculated as (percentage of induced apoptosis – percentage of spontaneous apoptosis)/(100 – percentage of spontaneous apoptosis).

2.16 Cell-cycle Analysis

Cell-cycle distribution was determined by the Propidium iodide (PI)-mediated flow cytometric analysis. PI is an intercalating agent that binds to nucleic acids and stains DNA quantitatively. Thus, by using this assay, different phases of the cell-cycle (G1, S, and G2) can be determined, as the fluorescence of cells in the G2 phase will be twice as high as that of cells in the G1 phase. In addition, sub-G1 population contains cells with lower DNA content and mostly reflects DNA fragmentation, which occurs in the late stage of apoptosis. Briefly, 5 × 10^5 cells were collected, fixed, and permeablized with cold 100% ethanol. After treatment with 10
μg/mL of DNase-free RNAse (Thermo Scientific), the cells were stained with 50 μg/mL of PI. Distribution of the cell-cycle phase with different DNA contents was determined with a FACSCalibur™ flow cytometer (BD Bioscience).

2.17 Immunofluorescence Staining and Confocal Microscopy

Cells (1 × 10⁵/per slide) were placed and adhered on poly-L-lysine coated slides (Electron Microscopy Sciences) for approximately 20 minutes at RT. Slides were then dipped into PBS to wash away the non-adherent cells. For BIN1 staining, cells were fixed with 4% paraformaldehyde for 20 minutes at RT and permeabilized by incubation with 0.05% Triton-X 100 in PBS for 5 min at RT. For Tubulin staining, cells were fixed and permeabilized with -20°C methanol for 10 minutes. For BIN1 and Tubulin double-staining, cells were first fixed in 4% paraformaldehyde for 20 minutes at RT and were further fixed and permeabilized with -20°C methanol for 10 minutes. Blocking was carried out for 20 minutes at RT with PBS containing 5% BSA. Cells were incubated with primary antibodies diluted in PBS-2% BSA for 2 hours at RT. The anti-BIN1 mouse monoclonal antibody (Abnova) was diluted at 1:200 ratio and the anti-α-tubulin rabbit polyclonal antibody (GeneTex) was diluted at 1:500 ratio in PBS-2% BSA. After washing with PBS (3 times), BIN1 and α-tubulin were detected by secondary antibodies conjugated to Alexa Fluor® 488 (Invitrogen) or Alexa Fluor® 594 (Invitrogen). Two negative controls (normal IgG control (Santa Cruz) and secondary-antibody-only control) were used to confirm the specificity of staining. DRAQ5 (Biostatus) or DAPI (Vector Laboratories) were used as nuclear stains and slides were viewed on a Nikon C1 confocal microscope or on an Olympus Fluoview 1200 confocal microscope.
2.18 Animals

Non-obese diabetic (NOD)/severe-combined immunodeficiency (SCID) mice were bred and maintained in microisolator cages provided with autoclaved food and water. For the first set of experiment, the mice at 8–10 weeks of age were injected subcutaneously with $20 \times 10^6$ parental HH or transduced-HH cells. While, for the second set of experiment, the mice at 8–10 weeks of age, were injected with $5 \times 10^6$ parental HH or transduced-HH cells. Mice were monitored for local tumor formation and tumor volume was measured regularly with external calipers. To determine the tumor volume by external caliper, the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were determined. Tumor volume was then calculated using the formula $V = (a \times b^2) / 2$, where “a” defines length (mm) and “b” width (mm) of the tumor (Euhus et al. 1986; Tomayko & Reynolds 1989). For histopathology, subcutaneous tumors and skin sections were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E), or with anti-human CD3 rabbit monoclonal antibody (Spring Bioscience) for immunohistochemical (IHC) staining. Histopathology analysis was performed by Centre for Translational and Applied Genomics (Vancouver). Images of histological slides were analyzed on a Zeiss Axioplan 2 Imaging microscope (Göttingen, Germany) equipped with a Retiga EXi colour digital camera (Burnaby, Canada). Animal experiments were performed in the Animal Resource Centre of BC Cancer Agency Research Centre, using procedures approved by the Animal Care Committee of the University of British Columbia (Vancouver).
2.19 Statistical Analysis

All data are shown as mean ± standard error of the mean (SEM) of measurements from at least three independent experiments. For most of the experiments, differences between groups were compared using the two-tailed Student’s t test for paired samples. Transcript levels in CTCL primary patient samples and controls were compared using the unpaired two-sample Student’s t test and the data are shown as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism version 6 (http://www.graphpad.com/scientific-software/prism/) and Microsoft Excel. P-values <0.05 were considered statistically significant.

X-Tile software, a marker cutpoint analysis tool developed by Camp et al., was applied to determine the optimal cut-point for BIN1 and c-FLIP expression levels as 1.36 and 411.1, respectively (Camp et al. 2004). In our analysis of disease-specific survival rate, BIN1 high and BIN1 low groups were defined by expression higher or lower than 1.36, respectively. c-FLIP high and c-FLIP low groups were defined by expression higher or lower than 411.1, respectively. Disease-specific survival rates were assessed using the Kaplan-Meier curves.
Table 2.1: Primary and secondary antibody conditions for western blotting

<table>
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<th>Antibody</th>
<th>Supplier</th>
<th>Primary Conditions</th>
<th>Secondary Conditions</th>
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<tr>
<td>Total BIN1 (monoclonal)</td>
<td>Abnova Cat.#: H00000274-M01</td>
<td>1:1,500 in 5% BSA</td>
<td>1:10,000 anti-mouse in 5% skim milk</td>
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<tr>
<td>BIN1 (12A) (monoclonal)</td>
<td>In house (from Dr. Prendergast)</td>
<td>1:1,000 in 5% BSA</td>
<td>1:10,000 anti-mouse in 5% skim milk</td>
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<td>Beta-Tubulin (polyclonal)</td>
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<td>Histone H3 (polyclonal)</td>
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<td>FLIP S/L (monoclonal)</td>
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<td>Caspase 8 (monoclonal)</td>
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<td>PARP (monoclonal)</td>
<td>Cell Signaling Technology Cat.#: 9542</td>
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<td>AHI-1 (N-terminal) (polyclonal)</td>
<td>Applied Biological Materials Inc. (Costume antibody production)</td>
<td>1:1,000 in 5% BSA</td>
<td>1:10,000 anti-rabbit in 5% skim milk</td>
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<td>GAPDH (monoclonal)</td>
<td>Sigma Aldrich Cat.#: G8795</td>
<td>1:1,000 in 5% BSA</td>
<td>1:10,000 anti-mouse in 5% skim milk</td>
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**Table 2.2: Primer sequences used in nested PCR**

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<th>Gene</th>
<th>Primer Sequence (PCR1)</th>
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<tr>
<td>BIN1 promoter</td>
<td>Fwd 5′-ATA ACA CCT ACC TCT AAA ACC AC -3′</td>
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<td></td>
<td>Rv   5′-TTG TTT TTT AAA GGG TTA TTT -3′</td>
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**Table 2.3: Primer sequences used for qRT-PCR**

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<tr>
<td>BIN1</td>
<td>Fwd 5′-GCA ACG TGC AGA AGA AGC TCA C -3′</td>
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<td></td>
<td>Rv 5′-GCT CAA ACT GCT CAT CCT TGG TC -3′</td>
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<tr>
<td>BIN1 12A</td>
<td>Fwd 5′-CGT CCA AGG AAG TCA AGC AGG -3′</td>
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<tr>
<td></td>
<td>Rv 5′-GGG TGG TCA CGC TGA TCT CAG -3′</td>
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<td>Fas</td>
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<td></td>
<td>Rv 5′-AGA AGA AGA CAA AGC CAC CCC -3′</td>
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<td>FasL</td>
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<td>Rv 5′-CCA GAG GCA TGG ACC TTG AG -3′</td>
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</tr>
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Chapter 3: Investigating the Role of Two Key BIN1 Isoforms in CTCL

3.1 Introduction

Cutaneous T-cell lymphoma (CTCL) represents a heterogeneous group of non-Hodgkin lymphomas characterized by initial infiltration of the skin with clonally-derived malignant T-cells (Assaf et al. 2004; Klemke et al. 2006; Booken et al. 2008; Li et al. 2012). The main representatives of CTCL are the skin-associated Mycosis Fungoides (MF) and its leukemic variant Sezary Syndrome (SS), which account for about 75% of CTCL cases (Kari et al. 2003; Klemke et al. 2006; Olsen et al. 2007). Both MF and SS are characterized by monoclonal proliferation of small- to medium-sized mature memory CD4+CD45RO+ T-cells that commonly lack normal T-cell markers, such as CD26 and CD7 (Klemke et al. 2006; Hwang et al. 2008). Despite several recent gene expression studies of SS and MF primary samples, the pathogenesis of these diseases is poorly understood and effective diagnostic tools and targeted treatment options are lacking (Kari et al. 2003; van Doorn et al. 2004; Booken et al. 2008; Mao et al. 2008; van Doorn et al. 2009; Wang et al. 2011; Dulmage & Geskin 2013; Guenova et al. 2014). Increasing evidence indicates that accumulation of malignant T-cells in SS and MF is mainly due to the defects in apoptosis pathways, including the Fas/Fas ligand (Fas/FasL)–mediated apoptosis pathway (Dummer et al. 1995; van Doorn et al. 2002; Valente et al. 2006; Braun et al. 2007; Contassot et al. 2008; Hwang et al. 2008; Wu et al. 2009; Wang et al. 2011; Wu et al. 2011). However, the molecular basis for the apoptosis resistance of CTCL cells is not well understood.

We have recently demonstrated that expression of the Abelson helper integration site-1 (AHI-1) oncogene is significantly increased in primary CD4+CD7- Sezary cells from SS patients (Ringrose et al. 2006; Kennah et al. 2009). Bridging integrator 1 (BIN1) was identified by
microarray analysis of CTCL cells as one of the genes involved in AHI-1-mediated lymphomagenesis (Kennah et al. 2009; Esmailzadeh & Jiang 2011). BIN1 is a nucleocytosolic adaptor protein that was first identified through its interaction with MYC box regions (MB1 and MB2) of the MYC oncoprotein (Sakamuro et al. 1996). The BIN1 gene contains 19 exons and alternative splicing can yield more than 10 isoforms with diverse patterns of distribution in tissues, sub-cellular localization and protein interaction (Sakamuro et al. 1996; Wechsler-Reya et al. 1998; Elliott et al. 2000; DuHadaway et al. 2003; Kinney et al. 2008; Cassimere et al. 2009; Prokic et al. 2014). BIN1 attenuation has been described in solid cancers, including melanoma, neuroblastoma, breast, colon, and prostate cancers (Ge et al. 1999; Ge et al. 2000a; DuHadaway et al. 2003; Tajiri et al. 2003; Chang et al. 2007a; Prendergast et al. 2009; Prokic et al. 2014). Loss of heterozygosity or mis-splicing of BIN1 is also associated with poor cancer prognosis and increased metastasis (Ge et al. 1999; Ge et al. 2000a; Ge et al. 2000b; Ghaneie et al. 2007).

Some isoforms of BIN1 (e.g. BIN1 (+10, +13)) have anti-proliferative and pro-apoptotic roles, acting through caspase-independent pathways (Elliott et al. 1999; Galderisi et al. 1999; Elliott et al. 2000; Cassimere et al. 2009; Prendergast et al. 2009). However, aberrant splicing of exon 12A is also observed in tumor cell lines and generates isoforms that usually lack the tumor suppressor properties (Ge et al. 1999; Elliott et al. 2000; Pineda-Lucena et al. 2005; Prendergast et al. 2009). Nevertheless, the role of BIN1 and its isoforms in the regulation of normal hematopoiesis and lymphomagenesis remains unknown. We have shown that transcript levels of BIN1 isoforms are significantly lower in CD4+CD7- Sezary cells from patients with SS compared to CD4+ cells from normal individuals (Kennah et al. 2009). Furthermore, four isoforms of BIN1 have been identified in CTCL Hut78 cells, in primary CD4+CD7- cells from SS patients and CD4+ cells from normal controls. These four isoforms, based on the presence or
absence of exons 10, 12A-D, and 13 are termed as: BIN1 (+10, +12A, +13), BIN1 (+10, +12A, -13), BIN1 (+10, +13) and BIN1 (+10, -13) (Kennah et al. 2009). Biological effects of BIN1 isoforms on pathogenesis of SS remain unknown and their involvement in other types of CTCL has not been investigated.

In this study, I investigated the biological and molecular effects of two key BIN1 isoforms on CTCL cells using a lentiviral-mediated gene transduction system. Restored expression of the two BIN1 isoforms in CTCL cells reveals anti-proliferative and pro-apoptotic roles, through downregulation of c-FLIP, which significantly inhibits the tumorigenic activity of CTCL cells in vivo. I also provided new evidence that downregulation of BIN1 and upregulation of c-FLIP are common in both SS and MF patient samples. In addition, I demonstrated that high BIN1 and low c-FLIP mRNA levels correlate with a better disease-specific survival rate in SS patients. I have thus identified BIN1 and c-FLIP as new, critical regulators and potential therapeutic targets in CTCL.
3.2 Results

3.2.1 Two Key BIN1 Isoforms are Stabley Overexpressed/Restorated in CTCL Cells Using a Lentiviral-mediated Gene Transduction Model

To investigate the biological and molecular role of BIN1 in CTCL, two key BIN1 isoforms (BIN1 +10, +13) and (BIN1 +10, +12A, +13) were lentivirally transduced into Hut78 and HH cells, two CTCL cell lines derived from peripheral blood of patients with CTCL, using MPG/MPY vectors. As experimental controls, empty vector MPG or MPY were also transduced into Hut78 and HH cells. Single-positive cells (GFP* or YFP*) were FACS-purified and enhanced expression of specific BIN1 isoforms was confirmed in transduced cells by western blot analysis (Figure 3.1A & B). Two BIN1 antibodies were used for western blotting; one detects all isoforms of BIN1 and the other detects only the isoforms with inclusion of exon 12A. Further studies to investigate the sub-cellular localization of the two BIN1 isoforms and their functions in CTCL cells were conducted using these transduced-Hut78 and transduced-HH cells.
Figure 3.1: Lentiviral transduction of two key BIN1 isoforms into CTCL cells

(A) Transduced Hut78 cells (top) and transduced HH cells (bottom) were FACS-purified based on their expression of the GFP marker. Representative FACS images demonstrate the percentage of GFP⁺ cells after FACS purification. MPG = vector control-transduced cells, BIN1 = BIN1 (+10, +13)-transduced cells and 12A = BIN1 (+10, +12A, +13)-transduced cells. (B) Western blot analysis of two BIN1 isoforms in BIN1-transduced Hut78 cells (left) and BIN1-transduced HH cells (right). Anti-total BIN1 and anti-BIN1 (12A) antibodies were applied to detect specific BIN1 isoforms as indicated. GAPDH was used as a control for protein loading.
3.2.2 BIN1 Isoforms are Mainly Localized in the Nucleus of Transduced CTCL Cells

It is known that BIN1 is a complicated adaptor protein with more than ten isoforms. Some of these isoforms are located in the cytoplasm, whereas others reside in the nucleus (Sakamuro et al. 1996; Wechsler-Reya et al. 1998; Prendergast et al. 2009). Based on their localization, these isoforms can carry out different functions in cells. Particularly, it has been reported in solid-tumor models that nuclear-localizing isoforms of BIN1 have tumor suppressor activities that can restrict proliferation, survival, and immune escape of oncogenically transformed cells (Elliott et al. 1999; Galderisi et al. 1999; Elliott et al. 2000; Cassimere et al. 2009; Prendergast et al. 2009). Interestingly, it has also been suggested that isoforms with inclusion of exon 12A are mainly localized to the cytoplasm and have no tumor suppressor properties in some solid tumor cell lines (Ge et al. 1999; Elliott et al. 2000; Pineda-Lucena et al. 2005; Prendergast et al. 2009). However, the localization of BIN1 isoforms in malignant hematopoietic cells, including CTCL cells, has not been explored. Thus, it is important to initially investigate the sub-cellular localization of BIN1 isoforms in CTCL cells. Since Hut78 cells express endogenous BIN1, whereas HH cells have no endogenous BIN1 expression, BIN1-transduced HH cells were used to further investigate sub-cellular localization of the two BIN1 isoforms. Interestingly, BIN1 (+10, +13) isoform was found to localize to both the nucleus and cytoplasm, with the majority in the nucleus, whereas BIN1 (+12A) isoform is exclusively localized to the nucleus, using sub-cellular fractionation followed by western blot analysis (Figure 3.2A). These results were further supported by immunofluorescence (IF) staining followed by confocal microscopy (Figure 3.2B). These results indicate that the two BIN1 isoforms are mainly localized to the nucleus and may have similar biological functions to restrict proliferation and survival in CTCL cells.
**Figure 3.2: Sub-cellular localization of BIN1 isoforms in BIN1-transduced CTCL cells**

(A) Western blot analysis of sub-cellular localization of BIN1 isoforms in transduced HH CTCL cells. Cells were fractionated into cytoplasmic (C) and nuclear (N) fractions followed by western blotting. Two BIN1 antibodies were used as indicated and Tubulin and Histone H3 antibodies were used as markers for detection of the cytoplasmic and nuclear fractions, respectively. **(B)** The same transduced cells were stained with anti-total BIN1 antibody and analyzed by Nikon C1 confocal microscope. Representative images at maximum magnification are shown. BIN1-transduced cells are labeled green (Alexa Fluor 488), and nuclei are stained with DRAQ5 (blue signal). Scale bars indicate 5 μm.
3.2.3 Restored BIN1 Expression in CTCL Cells Significantly Reduces Cell Proliferation and Increases Spontaneous Apoptosis

Several studies have demonstrated that restored expression of nuclear-localized isoforms of BIN1 reduces the cell growth and colony formation in different cell line models of solid tumors (Sakamuro et al. 1996; Elliott et al. 1999; Ge et al. 1999; Prendergast et al. 2009). Thus, to investigate whether restoration of expression of BIN1 isoforms in HH cells or stable overexpression of BIN1 isoforms in Hut78 cells has any effect on cell proliferation rate, [\(^{3}\text{H}\)]-thymidine incorporation assay was conducted on these cells. This assay measures the incorporation of [\(^{3}\text{H}\)]-thymidine, a radiolabeled DNA precursor, into the replication strands of DNA produced during cell division. Using this assay, a significant decrease in cell proliferation was observed in BIN1-trasduced-HH cells containing the two different isoforms and cultured in serum-containing media, as compared to empty-vector controls (~70% reduction after 48 hours, \(P < 0.01\), Figure 3.3). Similar results were observed in BIN1-transduced Hut78 cells in serum-free media but not in serum-containing media, possibly due to low levels of endogenous expression of other BIN1 isoforms in these cells (Figure 3.3).

To further investigate the effects of BIN1 isoforms on the ability of Hut78 and HH cells to proliferate \textit{in vitro}, we assessed their ability to form colonies in semi-solid media using a CFC assay. CFC output was also significantly decreased in transduced cells compared to empty-vector controls \((P \leq 0.009\), Figure 3.4A\). BIN1-transduced HH cells showed a greater reduction in CFC numbers than BIN1-tranduced Hut78 cells and produced smaller colonies compared to controls (70% vs. 50% reduction, Figure 3.4A-C).
Figure 3.3: Significant reduction in cell proliferation in BIN1-transduced CTCL cells

Percentage of $[^3\text{H}]$-Thymidine uptake in BIN1-transduced Hut78 cells (left) in the serum-free media and BIN1-transduced HH cells (right) in the serum-containing media after 24, 48, 72, and 96 hours. An asterisk indicates a significant difference between control and BIN1-transduced cells at *$P< 0.01$, and **$P< 0.001$. Data shown are the mean ± SEM of measurements from three independent experiments.
**Figure 3.4: Significant reduction in CFC output in BIN1-transduced CTCL cells**

(A) BIN1-transduced Hut78 cells (left) and BIN1-transduced HH cells (right) were plated in CFC assays. HH cells were plated in the serum-containing methylcellulose and Hut78 cells were plated in the serum-free methylcellulose cultures. Colonies produced were counted after 14 days of incubation, and the numbers obtained were expressed as a percentage of values obtained in vector control cells. (B) Numbers of CFCs based on the size of the colonies (small (<50), medium (50-500) and large (>500)) are indicated. (C) Representative images of the size and morphology of colonies in BIN1-transduced HH cells are shown. Data shown are the mean ± SEM of measurements from three independent experiments.
Furthermore, we studied the effect of BIN1 isoforms on cell cycle distribution in CTCL cells by conducting the PI cell-cycle assay. PI is an intercalating agent that binds to nucleic acids and stains DNA quantitatively. Thus, by using this assay, different phases of the cell-cycle (G1, S, and G2) can be determined, as the fluorescence of cells in the G2 phase will be twice as high as that of cells in the G1 phase. In addition, sub-G1 population contains cells with lower DNA content and mostly reflects DNA fragmentation, which occurs in the late stage of apoptosis. Interestingly, BIN1-transduced Hut78 cells showed significant increases in the apoptotic sub-G1 phase compared to the empty-vector controls (P≤ 0.05), whereas there were no significant differences in G1, S, or G2 phases (Figure 3.5). However, the PI cell-cycle assay cannot detect cells in earlier stages of apoptosis and thus the number of apoptotic sub-G1 population is usually lower than the number detectable with more accurate apoptosis assays. Therefore, the pro-apoptotic effect of BIN1 isoforms in CTCL cells was further confirmed by using the more accurate Annexin V-based apoptosis assay. Annexin V is a phospholipid-binding protein with high affinity for phosphatidylserine (PS), which in healthy cells resides in the inner side of the plasma membrane (Vermes et al. 1995). However, in the early stages of apoptosis, PS translocates from the inner side of the plasma membrane to the outer layer and becomes exposed at the external surface of the cell. Thus, unlike PI, Annexin V can be used as a probe for detecting the early stages of apoptosis before the cell membrane loses its integrity and DNA fragmentation occurs (Vermes et al. 1995). By performing the Annexin V-based apoptosis assay, we could further demonstrate a significant increase in spontaneous apoptosis in BIN1-transduced Hut78 and BIN1-transduced HH cells compared to controls after 24 hours (2-2.5-fold, P ≤ 0.04, Figure 3.6A & B). In conclusion, these results indicate that restored expression of BIN1 (+10, +13) and BIN1 (+10, +12A, +13) isoforms in CTCL cells, particularly in BIN1-
transduced HH cells, significantly reduces proliferation and increases apoptosis but has no significant effects on cell-cycle distribution.

**Figure 3.5: Significant increase in the apoptotic sub-G1 population in BIN1-transduced Hut78 cells**

BIN1-transduced cells were analyzed using the PI cell-cycle assay. Each cell-cycle is composed of G1, S, G2 stages, as well as a sub-G1 stage, which represents the apoptotic cells (left). Cell-cycle distribution is indicated as a percentage of three independent biological replicates. BIN1-transduced Hut78 cells revealed a significant increase in the sub-G1 population compared to controls, whereas there were no significant differences in G1, S, or G2 phases. Data shown are the mean ± SEM of measurements from three independent experiments.
Figure 3.6: Significant increase in spontaneous apoptosis in CTCL cells by resrotation of BIN1 expression

Percentage of total apoptotic cells in BIN1-transduced Hut78 in serum-free media (A) and BIN1-transduced HH cells in serum-containing media (B), including control cells, were determined after 24 hours by Annexin V/PI staining. Representative FACS images are shown. Data shown are the mean ± SEM of measurements from three independent experiments.
3.2.4 Restored BIN1 Expression Sensitizes CTCL Cells to the Fas/FasL-induced Apoptosis through Downregulation of c-FLIP

As described in Sections 1.2.3.2. and 1.3.4., one of the major pathways in maintaining the T-cell homeostasis is the Fas/FasL-mediated apoptosis pathway (Dhein et al. 1995; Ju et al. 1995; Sytwu et al. 1996; Gorak-Stolinska et al. 2001). Altered expression of different components of the Fas/FasL-mediated apoptosis pathway has been frequently observed in primary MF and SS patient samples, as well as in CTCL cell lines. Furthermore, deregulation of this pathway is thought to be critical in developing resistance to chemotherapeutic drugs in patients with CTCL (van Doorn et al. 2002; Kuzel 2003; Valente et al. 2006; Braun et al. 2007; Contassot et al. 2008; Wu et al. 2009; Wang et al. 2011; Wu et al. 2011). However, the molecular basis for the apoptosis resistance of CTCL cells remains largely unknown. Therefore, I further assessed whether the observed pro-apoptotic effects of BIN1 isoforms are specifically through the regulation of this apoptosis pathway. I initially examined the transcript levels of several key components of the Fas/FasL apoptosis pathway (Fas, FasL, and c-FLIP) in BIN1-transduced cells. Interestingly, a significant decrease in c-FLIP expression, a critical inhibitor of this pathway, was observed in BIN1-transduced cells compared to controls ($P \leq 0.04$), whereas no significant difference was detected for the Fas death receptor and its ligand FasL (Figure 3.7A). A decrease in the c-FLIP protein expression was further confirmed in both BIN1-transduced Hut78 and BIN1-transduced HH cells by western blot analysis (Figure 3.7B).

I next investigated whether the observed reduction in the c-FLIP expression by overexpression/restoration of BIN1 in transduced CTCL cells has any effect on their sensitivity to the Fas/FasL-mediated apoptosis and protein expression changes of downstream components of the pathway. I thus triggered this pathway by the addition of exogenous FasL (recombinant
FasL, 40ng/ml) in BIN1-transduced Hut78 cells, BIN1-transduced HH cells and their empty vector controls for 18 hours. Interestingly, BIN1-transduced Hut78 and BIN1-transduced HH cells displayed a significant increase in Fas/FasL-induced apoptosis compared to the empty-vector controls in response to the addition of exogenous FasL (2-3-fold, P < 0.005, Figure 3.8A). These changes were accompanied by an increase in the cleaved forms of caspase 8, caspase 3 and PARP, downstream proteins activated in the Fas/FasL-mediated pathway (Figure 3.8B).

**Figure 3.7: Significant decrease in the c-FLIP expression levels in CTCL cells by resrotation of BIN1 expression**

(A) qRT-PCR analysis of transcript levels of Fas, FasL and c-FLIP, after normalization to GAPDH, in transduced CTCL cells compared to vector-transduced control cells. Data shown are the mean ± SEM of measurements from three independent experiments. (B) Protein expression of c-FLIP (FLIP long (L) and short forms (S)) was analyzed in transduced cells by western blot analysis. GAPDH was used as a control for protein loading.
Figure 3.8: Sensitization of CTCL cells to the Fas/FasL-induced apoptosis and activation of the downstream caspase cascade by resrotation of BIN1 expression

(A) Percentage of the FasL-induced apoptosis in BIN1-transduced Hut78 cells (left) and in BIN1-transduced HH cells (right) compared to empty-vector controls after incubating cells with exogenous Super FasL (40 ng/mL) for 18 hours in culture. Data shown are the mean ± SEM of measurements from three independent experiments. (B) Protein expression of total caspase 8 (T. Casp8), cleaved caspase 8 (C. Casp8), total caspase 3 (T. Casp3), cleaved caspase 3 (C. Casp3), total PARP (T. PARP) and cleaved PARP (C. PARP) were analyzed in transduced cells by western blot analysis. GAPDH was used as a control for protein loading.

To further demonstrate that the observed increase in the Fas/FasL-induced apoptosis in BIN1-transduced CTCL cells is due to the downregulation of c-FLIP expression, I reintroduced c-FLIP into BIN1-transduced Hut78 and BIN1-transduced HH cells using the pMNDU3-pGK-eYFP (MPY) lentiviral vector. The overexpression of c-FLIP in BIN1-transduced cells was confirmed using western blotting (Figure 3.9A). Importantly, restored expression of c-FLIP into BIN1-transduced CTCL cells rescued the phenotypes, resulting in a significant decrease in the Fas/FasL-induced apoptosis (2-4-fold, $P < 0.05$; Figure 3.9B) and a significant reduction in protein expression of cleaved forms of caspase 8, caspase 3, and PARP (Figure 3.9A). In conclusion, these results indicate that the restored expression of BIN1 isoforms induces
apoptosis by downregulating the expression of c-FLIP, which mediates the Fas/FasL-induced apoptosis pathway in CTCL cells.

**Figure 3.9: Reduced sensitivity to the FasL-induced apoptosis in c-FLIP-restored cells with co-transduction of BIN1**

(A) The full-length c-FLIP was re-introduced into BIN1-transduced Hut78 and BIN1-transduced HH cells and its expression, as well as the caspase 8, caspase 3 and PARP expression were analyzed by western blot analysis compared to BIN1- and vector-control- transduced cells. (B) Percentage of the FasL-induced apoptosis in BIN1- and vector-control- transduced Hut78 (top) and HH cells (bottom), as well as in the BIN1-transduced Hut78 and HH cells co-transduced with c-FLIP after stimulation with exogenous Super FasL (40 ng/mL) for 18 hours in culture. Data shown are the mean ± SEM of measurements from three independent experiments.
3.2.5 Knockdown of BIN1 Isoforms in BIN1-transduced Cells Rescues their Effects on Cell Proliferation and Sensitivity to the Fas/FasL-induced Apoptosis

To further assess whether the effects on cell proliferation and apoptosis in BIN1-transduced cells are regulated directly by expression changes of BIN1, transient knockdown of BIN1 expression in BIN1-transduced Hut78 and BIN1-transduced HH cells was performed. This was achieved by using siRNA constructs containing two different targeting sequences against all isoforms of BIN1, including BIN1 (+12A). In addition, BIN1-transduced Hut78 and BIN1-transduced HH cells were transfected with a non-targeting control sequence and were used as experimental controls. Knockdown of BIN1 protein expression was confirmed by western blotting (>90%, Figure 3.10A). To investigate the effect of BIN1 suppression on cell proliferation of CTCL cells, the [3H]-thymidine incorporation assay was conducted. Interestingly, an increased proliferation was observed in these cells compared to scrambled controls after 24 hours (~2-fold, P ≤ 0.02; Figure 3.10B).

Next, the effect of BIN1 suppression on spontaneous apoptosis was assessed using the Annexin V-based apoptosis analysis. A large decrease in the number of apoptotic cells was observed in BIN1-suppressed Hut78 and BIN1-suppressed HH cells compared to controls after 24 hours (~2-fold, P ≤ 0.03; Figure 3.10C).
Figure 3.10: Enhanced cell proliferation and reduced apoptosis in BIN1 knockdown cells

(A) Western blot analysis of BIN1 expression in BIN1-suppressed and BIN1-transduced Hut78 cells (left) and BIN1-suppressed and BIN1-transduced HH cells (right), including scrambled-control cells. (B) Percentage of the [3H]-Thymidine uptake was measured relative to scrambled controls in BIN1-suppressed Hut78 cells (left) and BIN1-suppressed HH cells (right) after 24 hours in culture. (C) Spontaneous apoptosis was measured in BIN1-suppressed Hut78 cells (left) and BIN1-suppressed HH cells (right) after 24 hours compared to scrambled control cells. Data shown are the mean ± SEM of measurements from three independent experiments.
Moreover, both BIN1-suppressed Hut78 and BIN1-suppressed HH cells displayed a significant decrease in the Fas/FasL-induced apoptosis in the presence of exogenous FasL as compared to controls (2-3-fold, $P < 0.05$, Figure 3.11A). Thus, I further investigated whether this enhanced resistance to the Fas/FasL-induced apoptosis in BIN1-suppressed CTCL cells is due to alteration in c-FLIP expression. Strikingly, the c-FLIP protein expression was indeed upregulated in BIN1-transduced Hut78 and BIN1-transduced HH cells and subsequent decreases in the expression of cleaved forms of caspase 8, caspase 3, and PARP were detected in these cells as compared to controls (Figure 3.11B). These findings further support a critical role for BIN1 tumor suppressor activity in the regulation of anti-proliferative and pro-apoptotic activities of CTCL cells, particularly in the Fas/FasL-mediated apoptosis through c-FLIP.
Figure 3.11: Knockdown of BIN1 isoforms reduces sensitivity to the Fas/FasL-induced apoptosis in CTCL cells

(A) Percentage of the FasL-induced apoptosis in BIN1-suppressed Hut78 cells (left) and BIN1-suppressed HH cells (right) after the addition of exogenous Super FasL (40 ng/mL) and 18 hours in culture, compared to their scrambled control cells. Data shown are the mean ± SEM of measurements from three independent experiments. (B) Western blot analysis of protein expression of c-FLIP, Casp8, Casp3, and PARP in BIN1-transduced and BIN1-suppressed Hut78 (left) and HH cells (right) compared to scrambled control cells after the addition of exogenous Super FasL and 18 hours in culture. Antibodies are indicated and GAPDH serves as an internal loading control.
3.2.6 Restored BIN1 Expression in CTCL Cells Results in a Loss of the Ability to Induce Tumors in Immunodeficient mice

So far, I have provided strong evidence that restored expression of two key BIN1 isoforms inhibits proliferation and induces apoptosis in CTCL cells, including the Fas/FasL-mediated apoptosis through c-FLIP, using various in vitro assays. It would then be important to further assess whether the restoration of BIN1 isoforms has any impact on the ability of CTCL cells to produce tumors in vivo. Thus, the lymphomagenic potential of BIN1-transduced HH cells was compared to parental and empty-vector control HH cells by their subcutaneous injection into NOD/SCID mice. These mice were monitored for local tumor formation.

Initially, mice injected subcutaneously with either parental HH or control empty vector cells ($2 \times 10^7$ cells/per mouse) showed local tumor formation in six of six mice within 4 days post-injection. The local tumors enlarged progressively and were typically 1.5–2 cm in diameter by 2.5 weeks after injection. In contrast, no local tumors formed in mice given injections of equal numbers of BIN1-transduced HH cells after 14 days, in twelve of twelve mice. Tumor formation was only observed in BIN1-transduced HH cells after 3 weeks post-injection. However, the local tumors were significantly smaller in BIN1-transduced HH cells compared to controls (~3 folds). No tumor formation was observed in the PBS-injected control mouse (Appendix Figure A.1). In this experiment, all mice were euthanized within a month post-injection, due to rapid tumor formation. Thus, to optimize the in vivo conditions for monitoring tumor formation in mice for longer periods of time and also to confirm the results obtained, I set up another experiment by reducing the number of injected cells to $5 \times 10^6$ per mouse. In this experiment, mice injected with either parental HH or control empty-vector cells still showed aggressive tumor formation in six of six mice after 2 to 2.5 weeks (Figure. 3.12A). The local
tumors enlarged progressively and were typically 1.5–2 cm in diameter by 6 weeks post-injection (Figure 3.12A & B). In contrast, no local tumors formed in mice injected with the same numbers of BIN1-transduced HH cells, even after 8 weeks (total of twelve mice, Figure 3.12A & B).

Histological examination of skin tissues surrounding the subcutaneous tumors in HH- or empty-vector-injected mice showed epidermal infiltration of malignant CTCL cells, whereas no infiltration of tumor cells was observed in the skin sections of mice injected with BIN1-transduced HH cells or the PBS-injected control mice (Figure 3.13A). These infiltrated malignant CTCL cells were confirmed in positive control mice by staining tumor tissues with an antibody against human CD3 (Figure 3.13A & B), whereas there were no detectable malignant cells in mice injected with BIN1-transduced HH cells containing two different isoforms. These findings further demonstrate that restored BIN1 expression significantly inhibits the transforming activity of CTCL cells in vivo.
Figure 3.12: Inhibition of the transforming activity of CTCL cells by resoration of BIN1 expression *in vivo*

(A) Tumor volume comparison between NOD/SCID mice injected with BIN1-transduced HH cells containing two isoforms (n=6 for HHBIN1 and n=6 for HH12A) and controls (n=3 for HH cells and n=3 for HHMPG vector as positive controls; n=2 injected with PBS as negative controls). Mean tumor volumes ± SEM are shown. (B) Representative images of local tumor formation in HHMPG mouse but not in BIN1 mice, 6 weeks post-injection.
Figure 3.13: Histopathological analysis of skin tissues in mice injected with BIN1-transduced cells or control cells

(A) H&E staining of tissue sections showing tumor infiltration into skin section of the mouse injected with HHMPG cells and the absence of tumor infiltration in skin sections of mice injected with BIN1-transduced HH cells or with PBS alone (top, 5 × magnification). The presence of infiltrated malignant T cells in skin section of the mouse injected with HHMPG cells, as assessed by staining with a CD3 antibody (5 × magnification (middle) and 20 × magnification (bottom)). No CD3 could be detected in skin sections of mice injected with BIN1-transduced HH cells or PBS alone. (B) Representative images of dissected tumors from HHMPG mice (top) and the presence of malignant T cells in the tumor tissues by immunohistochemical staining with a CD3 antibody (bottom, 20 × magnification).
3.2.7 Transcript Levels of BIN1 and c-FLIP are Significantly Reduced or Upregulated in Patients with MF and SS

To investigate the transcript levels of total BIN1 and c-FLIP in primary samples from patients with MF and SS, qRT-PCR analysis was performed on mRNA samples extracted from skin biopsies of MF samples compared to controls with benign inflammatory dermatoses (atopic dermatitis) and CD4+CD7− cells from SS patients. Two sets of primers were used; one detects all isoforms of BIN1 and the other detects isoforms with inclusion of exon 12A (Figure 3.14A, Appendix Table A.1). Transcript levels of both total BIN1 and BIN1 (+12A) isoforms were significantly downregulated in MF patients (n = 10) compared to controls (n = 5, ~2-fold, \( P < 0.05 \), Figure 3.14A). Furthermore, transcript levels of total BIN1 in CD4+CD7− Sezary cells from PB of 13 patients with SS, were compared to CD4+ T-cells from controls (vitiligo = 3 and psoriasis = 3) and healthy donors (n = 5). As reported previously(Kennah et al. 2009), we consistently observed significantly downregulated expression of total BIN1 in CD4+CD7− Sezary cells from SS patients compared to normal controls (~3-fold, \( P < 0.05 \), Figure 3.14B). Interestingly, transcript levels of c-FLIP were significantly upregulated in both MF and SS patient samples as compared to controls (~2-fold, \( P < 0.05 \), Figure 3.14A & B). These results indicate that downregulated expression of BIN1 and upregulated c-FLIP is a common feature shared by patients with MF and SS.
Figure 3.14: Reduced transcript levels of BIN1 and increased levels of c-FLIP in primary MF and SS patient samples

(A) The transcript levels of total BIN1, BIN1 (+12A) isoforms and c-FLIP measured by qRT-PCR, normalized to GAPDH, from mRNA samples extracted from skin biopsies in patients with Mycosis Fungoides (MF, n = 10) compared to control samples with benign atopic dermatitis (AD, n = 5 for BIN1 and n = 4 for c-FLIP). Values shown are the mean ± standard deviation (SD) of measurements from control individuals and MF patients. 

(B) The transcript levels of total BIN1 (left) and c-FLIP (right) evaluated by qRT-PCR, normalized to GAPDH, in CD4+CD7− primary leukemic cells from 13 SS patients, 6 CD4+ from benign inflammatory dermatoses (BID, vitiligo = 3, psoriasis = 3) and 5 CD4+ from normal controls (N). Values shown are the mean ± SD of measurements from control individuals and SS patients.
3.2.8 High BIN1 and Low c-FLIP mRNA Levels Correlate with Increased Disease-specific Survival Rate in Patients with SS

To investigate whether there is any correlation between expression levels of BIN1 and c-FLIP and disease-specific survival rate, Kaplan-Meier analysis was performed on 12 patients with SS, for whom clinical outcome data are available (median follow-up time = 50 months, Appendix Table A.2). Although the number of SS patient samples was limited (n = 12), we detected a strong correlation between the high BIN1 and low c-FLIP expression levels and increased disease-specific survival rate (Figure 3.15). These results further support our hypothesis that BIN1 deficiency and the subsequent upregulation of c-FLIP may play an important role in CTCL pathogenesis and affect disease outcome.

Figure 3.15: High BIN1 and low c-FLIP mRNA levels correlate with a better disease-specific survival rate in patients with SS
Kaplan-Meier plot showing the correlation between BIN1 (left) and c-FLIP (right) mRNA levels with disease-specific survival rate in 12 patients with SS.
3.3 Discussion

This study provides the first evidence of strong tumor suppressor activity of BIN1 and its isoforms in primary CTCL cells and in CTCL cell model systems in vitro and in vivo. In particular, I have demonstrated that restored expression of BIN1 has strong anti-proliferative and pro-apoptotic roles in CTCL cells in vitro and can significantly inhibit the tumorigenic activity of these cells in vivo (Figure 3.3-3.6 & 3.12-3.13). I further showed that the pro-apoptotic role of BIN1 isoforms in CTCL cells occurs through downregulation of c-FLIP, which leads to activation of the Fas/FasL-mediated apoptosis pathway and its downstream caspase cascade (Figure 3.7-3.11). Thus, the loss of tumor suppressor activity of BIN1 contributes to enhanced proliferation and apoptosis resistance of CTCL cells through mediation of c-FLIP, enhancing transforming activity of these cells in mice. Importantly, I further showed a significant reduction in BIN1 transcript levels in both MF and SS patient samples compared to controls, while c-FLIP levels were significantly upregulated in these patients (Figure 3.14A & B). Interestingly, despite a relatively small sample size of SS patients (n = 12), I found a strong correlation between mRNA levels (high BIN1 and low c-FLIP) and a better disease-specific survival rate in SS patients (Figure 3.15).

BIN1 function is complex due to its alternative splicing and diverse patterns of distribution, sub-cellular localization and protein interaction (Sakamuro et al. 1996; Wechsler-Reya et al. 1998; Elliott et al. 2000; DuHadaway et al. 2003; Kinney et al. 2008; Cassimere et al. 2009; Prendergast et al. 2009; Prokic et al. 2014). Particularly, it has been reported that nuclear-localizing isoforms of BIN1 have tumor suppressor activities that can restrict proliferation, survival, and immune escape of oncogenically transformed cells (Elliott et al. 1999; Elliott et al. 2000; Muller et al. 2005; Cassimere et al. 2009; Prendergast et al. 2009).
It has also been suggested that aberrant splicing of neuron-specific exon 12A, in the absence of the other three neuron-specific exons (12B-D), yields a cancer-specific isoform that partially abolishes the tumor-suppressor activity of BIN1 in malignant cell lines from solid tumors, possibly by interfering with MYC binding (Ge et al. 1999; Elliott et al. 2000; Pineda-Lucena et al. 2005; Prendergast et al. 2009). I have investigated the sub-cellular localization of the ubiquitous BIN1 (+10, +13) and its potential cancer-specific isoform (+10, +12A, +13) in BIN1-transduced HH cells using two different techniques, sub-cellular fractionation and IF staining. The BIN1 (+10, +13) isoform localized to the nucleus and cytoplasm of HH cells, with the majority in the nucleus (Figure 3.2). This result is consistent with previous studies in solid tumors (Sakamuro et al. 1996; Elliott et al. 1999; Ge et al. 1999; Prendergast et al. 2009). Notably, the BIN1 (+10, +12A, +13) isoform exclusively localizes in the nucleus, with no detectable signal in the cytoplasm. Although the BIN1 (+12A) isoform has been observed to mainly localize in the cytoplasm in malignant cells (Wechsler-Reya et al. 1997; Prendergast et al. 2009), it has also been shown that retrovirally overexpressed BIN1 isoforms with inclusion of exon 12A are located in both the cytoplasm and nuclear fractions in human 293T cells and MCF-7 cells (human breast carcinoma cell line) (Pineda-Lucena et al. 2005). These results suggest that sub-cellular localization of 12A-containing isoforms is tissue-specific and that their biological functions and localization may alter in specific tissues and cell types from different malignancies. This hypothesis is further supported by demonstrating that transcript levels of BIN1 (+12A) isoform are significantly reduced in patients with MF and that its restored expression in CTCL cells reveals anti-proliferative and pro-apoptotic roles in vitro and in vivo, which is similar to the effects observed from the ubiquitous BIN1 isoform. These results are consistent with our previous observation that BIN1 (+12A) isoform was not only detected in
primary CD4^+CD7^- SS cells, but also in CD4^+ T-cells from healthy donors (Kenhah et al. 2009), suggesting that it is not a cancer-specific isoform in CTCL. In addition, I did not observe any expression changes in the MYC protein levels in BIN1 (+10, +12A, +13)-transduced cells (Appendix Figure A.2). Thus, I provide new information that the BIN1 (+10, +12A, +13) isoform is localized in the nucleus and has a strong tumor suppressor activity in human CTCL cells.

Apoptosis and cell death are essential in the control of lymphocyte homeostasis. Several lines of evidence propose that the pathogenesis of CTCL depends primarily on resistance to apoptosis rather than increased proliferation to account for the accumulation of tumor cells over time. In general, CTCL initiates as flat patches and thin plaques rather than bulky tumors. Early-stage CTCL is typically indolent until patients develop tumors or there is visceral involvement (Diamandidou et al. 1996; Siegel et al. 2000; Wu et al. 2009). Usually, tumor cells in the earlier stages of CTCL display a low proliferation rate and rarely demonstrate apoptosis (Dummer et al. 1995; Nevala et al. 2001). Consistent with these observations, patients with SS and MF are generally resistant to chemotherapy that targets rapidly dividing cells (Kuzel 2003). Thus, identifying new therapeutic targets that lead to apoptotic resistance in these patients has become one of the major aspects of CTCL research. Recently, several reports have consistently demonstrated that primary CTCL cells and cell lines are highly resistant to the Fas-mediated apoptosis pathway through deregulation of its key components such as, Fas, FasL, and c-FLIP (Meech et al. 2001; Braun et al. 2007; Contassot et al. 2008; Wu et al. 2009; Wang et al. 2011). In this study, I have demonstrated that restoration of expression of BIN1 and its isoforms triggers spontaneous apoptosis and specifically downregulates the c-FLIP expression and sensitizes these cells to the Fas/FasL-induced apoptosis, which induces subsequent activation of the caspase
cascade; these phenotypes can be rescued by reintroduction of c-FLIP or knockdown of BIN1 expression. Furthermore, I have observed a significant increase in c-FLIP transcript levels in primary MF and SS patient samples and identified a correlation between low c-FLIP mRNA levels and a better disease-specific survival rate in SS patients. Interestingly, increased expression of c-FLIP has been observed in other types of cancers, including non-Hodgkin’s lymphoma (Valente et al. 2006), Burkitt’s lymphoma (Valnet-Rabier et al. 2005), ovarian (Park et al. 2009; El-Gazzar et al. 2010), breast (Day et al. 2008), and pancreatic cancers (Haag et al. 2011), and melanoma (Yang et al. 2007). Other studies suggest that upregulation of c-FLIP protein may cause resistance to multiple anti-cancer drugs, since suppressing c-FLIP restores apoptosis triggered by death ligands, cytokines, and chemotherapeutic agents (Day et al. 2006; Safa et al. 2008; Logan et al. 2010; Safa & Pollok 2011). Hence, c-FLIP appears to be an attractive target for improved cancer treatment, including CTCL.

In addition, several studies have investigated the biological effects of Bin1 suppression in primary transformed MEFs as well as in Bin1 knockout mouse models. Interestingly, targeted deletion of Bin1 in transformed MEFs resulted in increased cell proliferation in vitro and producing large local tumors in vivo (Muller et al. 2004). Furthermore, based on two studies using mouse models with mammary gland-specific Bin1 deletion as well as mosaic Bin1 null mice, the important role for Bin1 as a negative modifier of oncogenicity and cancer susceptibility was further highlighted (Chang et al. 2007a; Chang et al. 2007b). In this study, to investigate the effect of BIN1 isoforms on lymphomagenic potential of CTCL cells in vivo, I used a common CTCL xenograft mouse model (Ringrose et al. 2006; Chen et al. 2009; Ito et al. 2009; Krejsgaard et al. 2010; Marchi et al. 2010), which is to subcutaneously inject CTCL cells into an immunodeficient mouse and monitor tumor formation. Using this model, I have shown that
subcutaneous injection of either parental or empty-vector control HH cells into NOD/SCID mice resulted in aggressive tumor formation. In contrast, subcutaneous injection of BIN1-transduced cells resulted in significantly smaller tumors or no tumor formation, based on the number of injected cells. These findings further support the negative effect of BIN1 isoforms on tumorigenic activity of CTCL cells in vivo.

In conclusion, I have demonstrated that the two key BIN1 isoforms are mainly localized to the nucleus and have strong anti-proliferative and pro-apoptotic roles in human CTCL both in vitro and in vivo. Loss/downregulation of these two isoforms in CTCL plays important roles in inducing apoptosis resistance by mediating c-FLIP expression and activation of its downstream caspase cascade. It is currently unknown how BIN1 regulates c-FLIP expression at molecular level and additional studies will be needed to elucidate direct or indirect interaction of these two proteins or additional proteins involved in this pathway. Nevertheless, this study has now identified BIN1 and c-FLIP as new, important regulators and potential therapeutic targets in CTCL. Thus, this study suggests that direct targeting c-FLIP activity or/and restoring BIN1 expression in CTCL cells may represent new strategy for overcoming apoptosis resistance, leading to more effective therapies for CTCL.
Chapter 4: Investigating the Potential Link between BIN1 and AHI-1 in CTCL Cells and Identifying New BIN1-interacting Proteins

4.1 Introduction

As described in Sections 1.4.2. and 1.5.2.1, transcript levels of the AHI-1 oncogene are highly elevated in CTCL cell lines (Hut78 and Hut102), as well as in primary CD4+CD7+ Sezary cells (Ringrose et al. 2006; Kennah et al. 2009). Furthermore, stable knockdown of endogenous AHI-1 in Hut78 cells reduces their transforming activity, both in vitro and in vivo (Ringrose et al. 2006). By conducting microarray analysis of AHI-1-suppressed Hut78 cells, several differentially expressed genes have been identified that may play critical roles in AHI-1-mediated leukemic transformation in CTCL (Kennah et al. 2009). One candidate gene is BIN1, which is upregulated at the RNA and protein levels in AHI-1-suppressed Hut78 cells and is downregulated in primary Sezary cells. In addition, overexpression or suppression of AHI-1 mediates expression changes of BIN1 in Hut78 cells (Kennah et al. 2009). These findings suggest that AHI-1 and BIN1 may be associated at the cellular and molecular levels to mediate the transforming properties of CTCL cells. In particular, AHI-1 may regulate expression of BIN1 and causes the latter to lose its tumor suppressor activity in altering cell proliferation and control of apoptotic pathways in human CTCL. Therefore, more detailed molecular and biological studies on these two genes may lead to important insights into the pathogenesis of this disease.

Thus, the aim of this chapter is to investigate how aberrant expression of AHI-1 leads to downregulation of BIN1 in CTCL cells at transcriptional and/or protein translational levels. I
have first investigated whether BIN1 physically interacts with AHI-1 in BIN1-transduced CTCL cells using co-IP/mass spectrometry analysis. Additionally, I have also used the same approach to identify new BIN1-interacting proteins and validated the results by co-localization studies. I have then tested the hypothesis whether AHI-1 affects the methylation status of the BIN1 promoter, which leads to its reduced gene expression in CTCL cells. Finally, I have investigated the biological effect of BIN1 overexpression on cell proliferation and apoptosis in AHI-1-suppressed CTCL cells.

In this study, there is no direct physical interaction between BIN1 and AHI-1 to be identified in CTCL cells by co-IP/mass spectrometry studies. My preliminary results have shown that the BIN1 basal promoter is mainly unmethylated in parental and BIN1-transduced Hut78 cells and differential expression of AHI-1 has no significant effect on altering this methylation status, although methylation analysis of the other regulatory regions of BIN1 has not been explored. Thus, the molecular mechanism by which AHI-1 regulates BIN1 expression still remains unknown. Nevertheless, several potential BIN1-interacting proteins were uncovered in CTCL cells, including α/β-tubulin and β-actin. In particular, co-localization of BIN1 and α-tubulin has been demonstrated in CTCL cells using confocal microscopy. Hence, further characterization of these newly identified proteins and their functional association with BIN1 may provide valuable insights into mechanisms underlying the CTCL pathogenesis.
4.2 Results

4.2.1 Investigating the Potential Protein Interaction between BIN1 and AHI-1 and Identifying BIN1-interacting Proteins

AHI-1 is an adaptor protein that can directly interact with multiple kinases to mediate leukemic cell transformation and drug resistance (Zhou et al. 2008; Chen et al. 2013). It contains several domains including a SH3 domain, a WD40-repeat domain and multiple SH3 binding sites (proline-rich domains, PxxP) (Figure 1.5) (Jiang et al. 2002; Esmailzadeh & Jiang 2011). Interestingly, BIN1 protein also contains a SH3 domain, and several SH3 binding motifs encoded by exons 10 and 12A (Figure 1.7) (Kojima et al. 2004; Pineda-Lucena et al. 2005). Thus, the presence of complimentary domains on these two proteins suggests a potential physical interaction between them. For instance, the SH3 domain of BIN1 may interact with the proline-rich domains (SH3 binding motifs) of AHI-1, or the proline-rich motifs of BIN1 may interact with SH3 domain of AHI-1. To further investigate this hypothesis, co-IP analysis was conducted on total cell lysates from control Hut78 cells as well as Hut78 cells transduced with the two isoforms of BIN1, BIN1 (+10, +13) and BIN1 (+10, +12A, +13). BIN1 was immunoprecipitated in protein lysates isolated from these cells, using the anti-total BIN1 mouse monoclonal antibody. Normal mouse IgG and Protein G PLUS-Agarose beads were used as two experimental negative controls to exclude any non-specific interactions. IP was followed by western blotting and the membrane was then probed with anti-AHI-1 rabbit polyclonal antibody. Although no interaction was detected between the full-length AHI-1 (~130 Kd) and BIN1, two bands were detected around 60 and 65 Kd in Hut78 and BIN1-transduced Hut78 cells that were absent from negative controls (Figure 4.1). These bands have similar molecular weights as the
shorter isoforms of AHI-1 (Jiang et al. 2004). To further verify these bands, BIN1 was immunoprecipitated and electrophoresed as described above and was stained with Coomassie® Blue. Interestingly, the same bands as the ones detected around 60 and 65 Kd with co-IP-western blotting were visualized with Coomassie® Blue staining in Hut78 and BIN1-transduced Hut78 cells, which were absent from the negative controls (Figure 4.2A).

In collaboration with Dr. Gregg Morin at the BCCA Genome Sciences Centre, mass spectrometry was performed to detect any potential protein interaction between BIN1 and the shorter isoforms of AHI-1 by analyzing these prominent bands at 60 and 65 Kd and their cognate slices from the negative control lanes. The candidate interacting proteins were selected based on the identification of at least two unique peptides for each protein and a Mascot score ≥ 50. In addition, the candidate interacting proteins were not found in control samples. To our surprise, no direct interaction between BIN1 and the shorter isoforms of AHI-1 was detected in Hut78 and BIN1-transduced Hut78 cells. However, several potential interacting partners for BIN1, with similar molecular weights (60-65 Kd), were identified in Hut78 and BIN1-transduced Hut78 cells, which are listed in Figure 4.2B. Furthermore, to identify other potential interacting proteins of BIN1 in CTCL cells, the other prominent bands (smaller and larger than 60-65 Kd) were also excised from BIN1 (+10, +12A, +13) and negative control lanes and were analyzed by mass spectrometry (Figure 4.2A & C). A list of these potential interacting partners for BIN1 (+10, +12A, +13) is shown in Figure 4.2C. Thus, although no direct interaction was identified between BIN1 and any isoforms of AHI-1, several other potential interacting partners for BIN1 were uncovered in CTCL cells including α/β-tubulin and β-actin. The potential interaction between BIN1 and α-tubulin was further investigated in Section 4.2.1.1., using confocal microscopy.
Figure 4.1: Co-immunoprecipitation studies between BIN1 and AHI-1 in CTCL cells

BIN1 was immunoprecipitated from protein lysates of control Hut78 and BIN1-transduced Hut78 cells and then electrophoresed and probed with anti-AHI-1 and anti-BIN1 antibodies, as indicated. Normal mouse IgG and Protein G PLUS-Agarose beads were used as negative controls. The red box indicates those bands around 60-65 Kd that are present in the Hut78 and BIN1-transduced Hut78 cells but are absent from negative controls. HutBIN1 = BIN1 (+10, +13) and Hut12A = (+10, +12A, +13).
## Protein Expression and Role

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cell line</th>
<th>Mascot score</th>
<th># Unique peptides</th>
<th>Role</th>
</tr>
</thead>
</table>
| BIN1                  | Hut78     | 689          | 15                | - More than ten isoforms  
- Nucleocytoplasmic adaptor proteins  
- Tumor suppressor activities; endocytosis; vesicle fusion and trafficking; actin organization; T-tubule formation |
|                      | HutBIN1   | 938          | 18                |                                                                       |
|                      | Hut12A    | 974          | 19                |                                                                       |
| β-Actin               | Hut78     | 218          | 6                 | - One of the essential components of the cytoskeleton  
- Ubiquitously expressed  
- Cell motility; cell division; regulation of gene expression |
|                      | HutBIN1   | 176          | 4                 |                                                                       |
|                      | Hut12A    | 179          | 5                 |                                                                       |
| α-Tubulin/β-Tubulin   | Hut78     | 207/72       | 5                 | - Basic structural units of microtubules  
- Key components of the cytoskeleton  
- Cell division and mitosis; cell signaling; transport of vesicles and other components throughout cells  
- Important targets for anticancer drugs |
|                      | HutBIN1   | 138/218      | 4                 |                                                                       |
|                      | Hut12A    | 168/129      | 4                 |                                                                       |
| NONO                  | Hut78     | 98           | 2                 | - RNA and DNA binding protein  
- Various roles in the nucleus: transcriptional regulation, RAN splicing, DNA winding and pairing |
|                      | Hut12A    | 169          | 4                 |                                                                       |
| Coronin-1A            | HutBIN1   | 103          | 3                 | - Actin binding protein  
- Exclusively expressed in hematopoietic lineages  
- Important roles during T lymphocytic activation, migration and survival |
Figure 4.2: Identification of potential interacting proteins of BIN1 in CTCL cells using immunoprecipitation followed by mass spectrometry

(A) Image of the Coomassie-stained gel, containing protein bands immunoprecipitated by mouse monoclonal anti-BIN1 antibody in control Hut78 and BIN1-transduced Hut78 cells, including HutBIN1 (+10, +13) and Hut12A (+10, +12A, +13) cells. (B) A list of potential BIN1 interacting proteins identified through mass spectrometry analysis of the gel bands indicated by red rectangles in section (A) (~60-65 Kd) in Hut78, HutBIN1 and Hut12A cells. These bands correlate to those detected around 60-65 Kd after the BIN1/AHI-1 co-IP/western blotting (Figure 4.1). (C) A list of potential BIN1 interacting proteins identified through mass spectrometry analysis of the gel bands indicated by yellow rectangles in section (A) in Hut12A cells.
4.2.1.1 Investigating the Potential Protein Interaction between BIN1 and α-tubulin

Interestingly, α/β-tubulin was identified as one of the potential interacting proteins of BIN1 in Hut78 and BIN1-transduced Hut78 cells through mass spectrometry. Indeed, several studies have demonstrated that BIN1 co-localizes with α-tubulin on cardiac T-tubules and regulates calcium channel trafficking by acting as an anchoring site for other proteins including Cav1.2 (Hong et al. 2010; Hong et al. 2014). In addition, it is important to note that I had previously observed a specific structure for BIN1 isoforms by conducting IF staining on HH and BIN1-transduced HH cells (Figure 3.2B), which resembled a cytoskeleton-related structures such as microtubules or actin filaments. Thus, to confirm the results from mass spectrometry, as well as to further characterize the specific structure of BIN1, I conducted IF staining for BIN1 and α-tubulin in Hut78 and BIN1-transduced Hut78 cells (BIN1 (+10, +13) and BIN1 (+10, +12A, +13)) using confocal microscopy. Single-staining of BIN1 in Hut78 and BIN1-transduced Hut78 cells, further confirmed the filament-like structures that were previously observed in HH cells (Figure 4.3). As described in Chapter 3 (Figure 3.1), Hut78 cells express endogenous levels of BIN1, as detected by western blotting. I have further confirmed this result by IF staining and visualizing the endogenous level of BIN1 in Hut78 cells, using an anti-total BIN1 antibody. Notably, the presence of filament-like structures of endogenous BIN1 in Hut78 cells indicates that these structures are real and are not artifacts generated by lentiviral transduction of BIN1 in these cells. These structures are similar to those of α-tubulin in Hut78 and BIN1-transduced Hut78 cells (Figure 4.4). Furthermore, I observed co-localization of BIN1 and α-tubulin in some areas in Hut78 and BIN1-transduced Hut78 cells (Figure 4.5). However, due to the lack of proper fixation method for double-staining of BIN1 and α-tubulin, the structures of these two proteins were mostly disrupted during the double-staining procedure. I have further discussed
the limitation of this method and alternative approaches in Section 4.3. Thus, based on these findings, it can be concluded that BIN1 and α-tubulin co-localize in some areas in Hut78 and BIN1-transduced Hut78 cells. However, an improved staining method or an alternative approach, such as co-IP of BIN1 and α-tubulin in control and BIN1-transduced CTCL cells, is needed to further confirm these results.

Figure 4.3: Sub-cellular localization of BIN1 isoforms in transduced Hut78 cells
BIN1 isoforms were stained with anti-total BIN1 antibody in empty-vector control and BIN1-transduced Hut78 cells (HutBIN1 and Hut12A) and analyzed by the Olympus Fluoview 1200 confocal microscope. Representative images at maximum magnification (60X) are shown. BIN1 isoforms are labeled red (Alexa Fluor® 594) and nuclei are stained with DAPI (blue signal).
Figure 4.4: Sub-cellular localization of α-tubulin in transduced Hut78 cells

Empty vector control and BIN1-transduced Hut78 cells were stained with anti-α-tubulin rabbit polyclonal antibody and analyzed by the Olympus Fluoview 1200 confocal microscope. Representative images at maximum magnification (60X) are shown. α-tubulin is labeled green (Alexa Fluor® 488) and nuclei are stained with DAPI (blue signal).
Figure 4.5: Sub-cellular localization of BIN1 and α-tubulin in transduced Hut78 cells
BIN1 isoforms were stained with mouse monoclonal anti-total BIN1 antibody and α-tubulin was stained with rabbit polyclonal anti-α-tubulin antibody in transduced Hut78 cells and analyzed by the Olympus Fluoview 1200 confocal microscope. Representative images at maximum magnification (60X) are shown. α-tubulin is labeled green (Alexa Fluor® 488), BIN1 is labeled red (Alexa Fluor® 594), and nuclei are stained with DAPI (blue signal).
4.2.2 Sub-cellular Localization of AHI-1 in CTCL Cells

In Chapter 3, I demonstrated that the BIN1 (+10, +13) isoform is mainly located in the nucleus with some cytosolic localization, whereas the BIN1 (+10, +12A, +13) isoform is exclusively located in the nucleus of CTCL cells. Localization of two proteins within the same sub-cellular fraction is potentially an indicator of their direct or indirect interaction and increases the possibility of their involvement in the same signaling pathway(s). Thus, to further assess the possibility of any interaction between BIN1 and AHI-1, I have also studied the sub-cellular localization of AHI-1 in CTCL cells and compared it to that of the BIN1 isoforms. Using sub-cellular fractionation, I have demonstrated that in contrast to the BIN1 isoforms, AHI-1 is mainly located in the cytoplasm in Hut78 and HH cells (Figure 4.6). Thus, in addition to the results from mass spectrometry in Section 4.2, the difference between the sub-cellular localizations of AHI-1 and BIN1 isoforms has further reduced the possibility of physical interaction between these two proteins in CTCL cells.
Figure 4.6: Sub-cellular localization of AHI-1 in BIN1-transduced CTCL cells and empty-vector controls

Western blot analysis of sub-cellular localization of AHI-1 in transduced Hut78 cells (left) and transduced HH cells (right). Cells were fractionated into cytoplasmic (C) and nuclear (N) fractions followed by western blotting. Tubulin and Histone H3 antibodies were used as markers for detection of the cytoplasmic and nuclear fractions, respectively.
4.2.3 Investigating the Effect of AHI-1 on Methylation Status of the BIN1 Promoter

BIN1 attenuation has been reported in several types of cancer including breast and prostate cancers (Ge et al. 2000a; DuHadaway et al. 2003; Prokic et al. 2014). However, genetic alterations and mutations are only found in a minority of these cases, suggesting that the loss of BIN1 is mostly due to epigenetic changes (Ge et al. 2000a; Kuznetsova et al. 2007). Interestingly, structural analysis has demonstrated that the human BIN1 promoter contains a CpG island (CGI) and hyper-methylated BIN1 has been reported in fractions of primary breast and prostate cancer samples (Kuznetsova et al. 2007; Barekati et al. 2012). In addition, hyper-methylation of the BIN1 promoter has been identified not only in tumor tissues but also in plasma and serum samples from patients with breast cancer (Radjpou et al. 2011). Since BIN1 transcript levels are downregulated in primary SS and MF samples and suppression or overexpression of the AHI-1 oncogene mediates expression changes of BIN1 in CTCL cells, I have hypothesized that a high expression of the AHI-1 oncogene leads to downregulation of the BIN1 tumor suppressor by regulating the methylation status of the BIN1 promoter region. BIN1 promoter CGI spans about 1650 bp of genomic sequence including the 5'-upstream region, exon 1 and a fragment of intron 1, and thus includes both promoter and non-promoter sequences (Kuznetsova et al. 2007). In addition, structural and functional analyses have shown that a 900-bp sequence upstream of the exon 1 contains consensus sites for several transcription factors and has a basal promoter activity (Wechsler-Reya et al. 1997). Thus, to test this hypothesis, I first extracted genomic DNA from four cell lines with different expression levels of AHI-1: parental Hut78, empty-vector-transduced Hut78 (RPG), AHI-1-suppressed Hut78 (AHI-1-sh4), and AHI-1-overexpressed Hut78 (Lenti-AHI-1) cells. The AHI-1-sh4 retroviral and the Lenti-AHI-1 constructs were previously generated by former members of our group (Ringrose et al. 2006;
Kehnah et al. 2009). I further used the same constructs to freshly transduce Hut78 cells and made new AHI-1-sh4 and Lenti-AHI-1 cell lines. The differential expression levels of AHI-1 in these cells were further confirmed by western blotting (Appendix Figure A.3). Subsequently, I tested the effect of differential expression levels of AHI-1 on methylation status of the BIN1 basal promoter by amplifying the 700-bp sequence upstream of exon 1 and conducting bisulfite sequencing (Figure 4.7A). The bisulfite sequencing results of the BIN1 basal promoter revealed that this region is mainly unmethylated in both AHI-1-suppressed and overexpressed Hut78 cells (AHI-1-sh4 and LentiAHI-1), as well as in controls (Hut78 and RPG) (1.7% - 8.8% methylation, Figure 4.7B & C), although relative high methylation status was observed in AHI-1-overexpressed cells (~8-12%). Thus, differential expression of AHI-1 has no significant effect on methylation status of the BIN1 basal promoter region in Hut78 cells.
Figure 4.7: Methylation analysis of the BIN1 basal promoter in CTCL cells
(A) Schematic drawing of the 5'-region of the BIN1 promoter region. The BIN1 promoter CpG island (CGI) covers exon 1, a fragment of intron 1 and most of the 0.9-kb basal promoter region. The red arrows indicate primers used in this study to amplify the CGI of the basal promoter. (B) Distribution of methylated (black circles) and unmethylated (white circles) CpG sites across the basal promoter of BIN1 in parental Hut78, empty-vector control (RPG), AHI-1-suppressed Hut78 (AHI-1-sh4-1 & -2) and AHI-1-overexpressed Hut78 (Lenti-AHI-1) cells. (C) Methylation percentage of the basal promoter of BIN1 was calculated based on bisulfite sequencing of at least five clones per cell line.
4.2.4 Investigating the Biological Effect of BIN1 Overexpression in AHI-1 suppressed CTCL cells

In Chapter 3, I demonstrated that overexpression or restoration of BIN1 expression in CTCL cells significantly reduces cell proliferation and increases apoptosis. These CTCL cells express endogenous levels of AHI-1. However, to further investigate a potential biological link between AHI-1 and BIN1, I first assessed whether BIN1 exhibits additional anti-proliferative and pro-apoptotic roles when it is overexpressed in AHI-1 suppressed cells, since the vector constructs and cell lines are available to use. To do so, I lentivirally transduced the BIN1 (+10, +13) isoform into AHI-1-suppressed cells (AHI-1-sh4 cells) and confirmed the overexpression of BIN1 in these cells by western blotting (Figure 4.8A). In addition, empty vector (MPY)-transduced AHI-1-sh4 cells were used as experimental controls. Notably, the endogenous levels of BIN1 isoforms were highly expressed in parental Hut78 and vector-transduced AHI-1-sh4 cells. Interestingly, in accordance with our previous observation (Ringrose et al. 2006), MPY-transduced AHI-1-sh4 cells displayed a significant reduction in the numbers of CFC output and an increase in the number of apoptotic cells as compared to parental Hut78 cells (Figure 4.8B). However, additional effects were not observed in BIN1-transduced AHI-1-sh4 cells compared to the MPY-transduced AHI-1-sh4 controls (Figure 4.8B). One possible reason could be due to the already high expression levels of endogenous BIN1 in these cells (Figure 4.8A). Several studies have reported that BIN1 overexpression in non-malignant cells or in cells with endogenous expression of BIN1 has no significant effect on altering the cell proliferation and apoptosis, possibly due to the high expression levels of endogenous BIN1 in these cells (Sakamuro et al. 1996; Elliott et al. 2000; DuHadaway et al. 2001). Therefore, it is possible that the anti-proliferative and pro-apoptotic effects that are observed in AHI-1-sh4 cells compared to parental
Hut78 cells is related to the high expression levels of endogenous BIN1 and thus, further increase in BIN1 levels does not enhance these phenotypes. The use of an inducible vector system or knockdown the endogenous level of BIN1 in AHI-1-sh4 cells may provide a better model system to elucidate the biological link between BIN1 and AHI-1 in CTCL.
Figure 4.8: Effects of BIN1 overexpression on cell proliferation and apoptosis in AHI-1 suppressed CTCL cells

(A) Western blot analysis of BIN1 in Hut78 cells, empty vector-transduced AHI-1-suppressed cells (AHI-1-sh4) and BIN1-transduced AHI-1-sh4 cells. GAPDH was used as a control for protein loading. (B) Hut78 cells, empty vector-transduced AHI-1-sh4 cells and BIN1-transduced AHI-1-sh4 cells were plated in CFC assays. Colonies produced were counted after 14 days of incubation, and the numbers obtained were expressed as a percentage of values obtained in Hut78 cells. (C) Percentage of total apoptotic cells in BIN1-transduced AHI-1-sh4 and control cells were determined after 24 and 48 hours of culturing cells in serum-free media, using Annexin V/PI staining. Data shown are the mean ± SEM of measurements from two independent experiments.
4.3 Discussion and Future Directions

In Chapter 4 of this thesis, I have extensively investigated potential biological and molecular links between BIN1 and AHI-1 in CTCL cells using a variety of assays. Although no direct physical interaction was identified between BIN1 and AHI-1, several other potential interacting proteins of BIN1 have been identified in CTCL cells, using co-IP/mass spectrometry analysis (Figures 4.1 & 4.2). Interestingly, several of these potential interacting proteins, including α/β-tubulin, β-actin and myosin have been previously reported to co-localize and physically interact with BIN1 in skeletal muscle cells and cardiomyocytes (Fernando et al. 2009; Hong et al. 2010; Hong et al. 2014). One study has reported that BIN1 can interact with actin and myosin through its SH3 domain and mediates sarcomere assembly and organization, which is an important step in skeletal muscle differentiation and myofiber maturation (Fernando et al. 2009). Furthermore, several studies have also demonstrated that BIN1 co-localizes with α-tubulin and α-actinin (an actin binding protein) on cardiac T-tubules and regulates calcium channel trafficking by acting as an anchoring site for other proteins including Cav1.2, as well as by regulating the T-tubule membrane folding (Lee et al. 2002; Hong et al. 2010; Hong et al. 2014). These functions of BIN1 are essential for regulating the strength of each heartbeat and limiting arrhythmia and heart failure in mice (Hong et al. 2014). Interestingly, restored expression of BIN1 in Hela cells and Chinese hamster ovary cells has been shown to increase the formation of tubular structures and subsequent increase in α-tubulin staining, indicating the intrinsic tubulating capacity of BIN1 (Lee et al. 2002; Meunier et al. 2009). In this study, I confirmed the mass spectrometry results by demonstrating the co-localization of BIN1 and α-tubulin in some areas of CTCL cells (Figure 4.5). However, in contrast to the single-staining procedure for either BIN1 or α-tubulin (Figures 4.3 & 4.4), the structures of these two proteins
were mainly disrupted during the double-staining procedure due to the lack of a proper fixation method that works well with both BIN1 and α-tubulin antibodies. The fixation step is needed to maintain cellular structure as close as possible to the native state. However, no fixative methods completely preserve the in vivo distribution of soluble proteins or proteins whose association with cellular structures is weak. Thus, finding a suitable fixation method can sometimes be challenging, especially for visualizing two or more proteins within a cell. In this study, 4% paraformaldehyde was the fixative of choice for staining BIN1 in CTCL cells. However, paraformaldehyde does not maintain microtubule integrity very well and thus 100% methanol was used as the fixative of choice for detecting α-tubulin in CTCL cells. Methanol denatures and extracts cytosolic and nuclear proteins (Schnell et al. 2012) and as I tested before, it destroys the BIN1 structure in CTCL cells. It has been previously shown that paraformaldehyde fixation before methanol permeabilization can markedly reduce denaturation of proteins (Hoetelmans et al. 2001; Schnell et al. 2012). Thus I conducted this double-fixation method to co-stain BIN1 and α-tubulin in CTCL cells. Although, using this technique has significantly improved the quality of the BIN1/α-tubulin co-staining, the loss of integrity of intracellular structures was still observed compared to the single-staining procedure. Therefore, further optimization is still needed for enhancing the quality of the BIN1/α-tubulin co-staining in CTCL cells. However, IF staining, using fixation and permeabilization methods, has some limitations including protein extraction or relocalization (Schnell et al. 2012). For example, Schnell et al. have investigated the sub-cellular localization of several proteins using two different methods: (1) immunostaining experiments in dead, permeabilized cells, and (2) live-cell imaging using fluorescent fusion proteins (Schnell et al. 2012). Having investigated the limitations and artifacts of each of these approaches, Schnell et al. recommended that immunostaining experiments in dead,
permeabilized cells should be complemented with live-cell imaging using tagged chimeras to avoid any misinterpretation of microscopy data (Schnell et al. 2012). Thus, using the fluorescent-fusion-BIN1 and -α-tubulin and conducting live cell imaging in parallel to the previously-conducted immunostaining experiments on fixed cells may further improve the quality of the microscopy images. This should further enhance the confidence in my conclusion that BIN1 and α-tubulin co-localize in CTCL cells. In addition to different microscopy imaging techniques, I will further carry out a co-IP assay using BIN1 and α-tubulin antibodies to further confirm the potential interaction between these two proteins in CTCL cells.

Nevertheless, identification of α/β-tubulin and β-actin as potential BIN1 interacting proteins may have interesting functional significance in CTCL cells. Interestingly, some studies have demonstrated a role for cytoskeleton proteins including α/β-tubulin and ubiquitous isoforms of actin (β-actin and γ-actin) in regulating apoptosis signaling (Moss et al. 2006; Desouza et al. 2012). The actin cytoskeleton has been shown to act as both the initiator and mediator of apoptosis (Desouza et al. 2012). The Fas-mediated apoptosis pathway has been indicated to be dependent upon the interaction of Fas receptor with actin via the actin-binding protein ezrin in CD4+ T-cells (Parlato et al. 2000; Desouza et al. 2012). Ezrin can anchor to the Fas receptor at the cell membrane and transduce an extracellular signal to the actin cytoskeleton and initiate apoptosis signaling (Algrain et al. 1993). Furthermore, studies in yeast have elucidated that the expression of a mutant form of actin with decreased actin dynamics increases susceptibility to mitochondrial apoptosis pathway, due to the accumulation of reactive oxygen species and mitochondrial membrane depolarization (Belmont & Drubin 1998). The role of actin filaments in regulating the mitochondrial apoptosis pathway has been further confirmed in mammalian cell lines (e.g. Hela and Jurkat cells), by treating these cells with inhibitors of actin polymerization
Moreover, it is known that dramatic changes in cellular dynamics characterize the apoptotic execution phase which occurs immediately after a cell commits to apoptosis (Mills et al. 1999). Early during the execution phase, apoptotic cells lose their focal contacts with the extra-cellular environment and undergo a transient period of surface blebbing (Mills et al. 1999). Ultimately, cell fragmentation occurs and apoptotic cells break up into sealed membrane vesicles, called apoptotic bodies, which are eventually eliminated by phagocytosis (Mills et al. 1999). Studies have also demonstrated the essential role of both actin and tubulin in membrane blebbing and formation of the apoptotic bodies, which are necessary steps for apoptosis to occur (Moss et al. 2006; Desouza et al. 2012). Furthermore, a study on HL-60 and U-937 cells has demonstrated that treating these cells with actin targeting drugs inhibits the formation of apoptotic bodies and increases apoptosis resistance (Cotter et al. 1992). Therefore, based on the following three factors: (1) the important roles of actin and tubulin in regulating apoptosis, (2) the role of BIN1 in actin cytoskeletal organization, as well as in microtubule formation and maintenance (described earlier in this section), and (3) the pro-apoptotic role of BIN1 in several types of cancer cells, including CTCL cells, I can hypothesize that BIN1 controls apoptosis through interaction with tubulin and actin and potentially regulates the cytoskeletal dynamics in CTCL cells. Hence, further characterization of these cytoskeleton proteins and their functional association with BIN1 can provide valuable insights into mechanisms underlying the CTCL development.

Furthermore, I have tested the hypothesis that aberrant expression of AHI-1 leads to downregulation of the BIN1 tumor suppressor through epigenetic changes. In particular, I assessed the effect of AHI-1 on methylation status of the BIN1 basal promoter, using bisulfite sequencing. The BIN1 promoter was found to be mostly unmethylated in CTCL cells and AHI-1
had no significant effect on altering this status. However, as described in Section 4.2.3, the BIN1 promoter CGI spans about 1650 bp of genomic sequence including the 5\'-upstream region, exon 1 and a fragment of intron 1, and include both promoter and non-promoter sequences (Kuznetsova et al. 2007). In general, it is known that the methylation status can be heterogeneous and affects different regions of a promoter CGI. For instance, the promoter CGIs of CDKN2A and RASSF1A genes have shown to be frequently and densely methylated at the 3\'-regions and to a lesser degree at the 5\'-regions in bladder and breast cancers, respectively (Tsutsumi et al. 1998; Yan et al. 2003). Furthermore, Kuznetsova et al. have demonstrated that the 3\'-region of the BIN1 promoter CGI is methylated in a fraction of samples from patients with prostate and breast cancers, whereas no methylation has been revealed in its 5\'-region in the same samples (Kuznetsova et al. 2007). Therefore, it would be of interest to investigate the methylation density of the core region and the 3\'-region of the BIN1 CGI in CTCL cells and to further explore the effect of AHI-1 on the methylation status of these areas.

In addition to promoter hypermethylation, chromatin remodeling has been proposed as another epigenetic mechanism to regulate BIN1 expression in cancer cells (McKenna et al. 2012). SWI/SNF chromatin remodeling multi-subunit complexes utilize the energy of ATP hydrolysis to mobilize nucleosomes, remodel chromatin, and modulate transcription of various target genes (Wilson & Roberts 2011). Mutation and altered expression of several subunits of the SWI/SNF complex have been reported in variety of cancers (McKenna et al. 2012). For instance, biallelic inactivation of SNF5, a core subunit of the SWI/SNF complex, has been identified in 98% of rhabdoid tumors (RT), an aggressive type of pediatric cancer (Versteeghe et al. 1998; McKenna et al. 2012). McKenna et al. have demonstrated that loss of SNF5 leads to downregulation of the BIN1 tumor suppressor in primary samples as well as in cell culture.
models of RT and that this downregulation is needed for the proliferation of SNF5-deficient tumor cells (McKenna et al. 2012). They have further shown that SNF5 recruits the SWI/SNF complex to the BIN1 promoter, and that the downregulation of BIN1 expression correlates with decreased SWI/SNF occupancy. Based on these recent findings, it would be of interest to further investigate whether the AHI-1 oncogene can regulate BIN1 expression through chromatin remodeling by altering the expression levels of SNF5 or other components of the SWI/SNF complex.

It has been shown that suppression and overexpression of AHI-1 result in upregulation and downregulation of the BIN1 transcript levels in CTCL cells, respectively (Kennah et al. 2009). These results suggest that AHI-1 may affect the transcript levels of BIN1 by negative regulation of the BIN1 promoter activity. Although AHI-1/Ahi-1 is an adaptor protein with no DNA-binding motifs, a study has demonstrated that it can facilitate the translocation of beta-catenin, an important transcription factor in the canonical Wnt signaling pathway (Lancaster et al. 2009). Wnt activity is upregulated in mouse renal injury and has been shown to have a role in adult renal homeostatic injury repair (Surendran et al. 2005; Lancaster et al. 2009). Lancaster et al. have demonstrated that the mouse Ahi-1 interacts with beta-catenin and facilitates its translocation and accumulation in the nucleus, resulting in positive modulation of downstream transcription (Lancaster et al. 2009). Therefore, Ahi-1 indirectly manipulates the expression levels of the beta-catenin target genes by acting as a shuttle protein. Hence, it would be interesting to further investigate whether AHI-1 has any effect on the promoter activity of BIN1 in CTCL cells. For example, luciferase reporter assay using a BIN1 promoter-driven reporter construct can be used in Hut78, AHI-1-sh4, and Lenti-AHI-1 cells to investigate the effect of different expression levels of AHI-1 on promoter activity of BIN1. Furthermore, chromatin
immunoprecipitation (CHIP) can be applied to identify potential BIN1 transcription factors in CTCL cells. Later, based on the role of AHI-1 as a shuttle protein, its potential effect on the translocation of BIN1 transcription factors in CTCL cells can be further investigated, using the IF staining or the sub-cellular fractionation methods.

In summary, I identified several cytoskeletal proteins, including β-actin and α/β-tubulin, as potential interacting partners of BIN1 in CTCL cells through co-IP/mass spectrometry. Potential protein interaction between BIN1 and α-tubulin has further been validated in transduced CTCL cells by sub-cellular localization studies using confocal microscopy. However, the significance and the role of these cytoskeletal proteins in CTCL pathogenesis are poorly understood. Thus, functional characterization of these cytoskeletal proteins and identifying their functional association with the tumor suppressor BIN1 may lead to a better understanding of the underlying molecular mechanisms of CTCL. A deeper insight into the roles of specific genes and signaling pathways involved in CTCL pathogenesis may eventually lead to identification of new molecular targets and more effective treatment options.
Chapter 5: General Summary and Future Directions

5.1 Summary

CTCL is considered to be a sporadic disease. The etiology and molecular pathogenesis of CTCL are largely unknown. During the last ten years, many genetic studies have been conducted on primary CTCL samples in order to reach one of the following aims: 1) to establish biomarkers for CTCL diagnosis, 2) to identify genes important to CTCL pathogenesis, and 3) to discover potential therapeutic targets (Dulmage & Geskin 2013). I believe I have made some useful contributions to this field. My work has added to our understanding of mechanisms involved in the development of apoptosis resistance in CTCL and has identified potential targets for improved CTCL treatment.

In Chapter 3, I mainly focused on investigating the role of BIN1 and its isoforms in the regulation of cell proliferation, apoptosis and tumor formation in human CTCL cells, using in vitro and in vivo assays. By means of in vitro assays, I demonstrated that overexpression/restored expression of BIN1 has strong anti-proliferative and pro-apoptotic roles by increasing both spontaneous and Fas/FasL-induced apoptosis in CTCL cells. I further showed that the pro-apoptotic role of BIN1 isoforms in CTCL cells occurs through downregulation of c-FLIP which leads to subsequent activation of the caspase cascade; and that these phenotypes were rescued by reintroduction of c-FLIP or knockdown of BIN1 expression. Moreover, subcutaneous injection of HH cells, with no endogenous expression of BIN1, led to an aggressive tumor formation in immunodeficient NOD/SCID mice. Conversely, restoration of BIN1 expression in these cells significantly inhibited their tumorigenic activities in these mouse
models. Hence, the loss of tumor suppressor activity of BIN1 contributes to enhanced proliferation and apoptosis resistance of CTCL cells through mediation of c-FLIP, enhancing the transforming activity of these cells in mice (Figure 5.1). I also provided new evidence that downregulation of BIN1 and upregulation of c-FLIP transcript levels are common in both SS and MF patient samples. In addition, I demonstrated that high BIN1 and low c-FLIP mRNA levels correlate with a better disease-specific survival rate in SS patients.

In addition, our group has previously identified BIN1 as a potential gene involved in the AH1-1-mediated lymphomagenesis in CTCL cells, using microarray analysis (Kenhah et al. 2009). However, how AH1-1 and BIN1 are associated in regulation of the transforming activity of CTCL cells had remained elusive. In Chapter 4, I described my research experiments to identify the potential link between these two genes. I investigated the protein interaction between BIN1 and AH1-1 by means of co-IP/mass spectrometry and sub-cellular fractionation studies. Although no direct physical interaction was detected between BIN1 and AH1-1, several other potential BIN1 interacting proteins were uncovered in CTCL cells, including α/β-tubulin and β-actin. The interaction between BIN1 and α-tubulin was further confirmed by confocal microscopy. Interestingly, both tubulin and actin have been reported to play major roles in regulating cell-cycle and apoptosis of malignant cells (Jordan & Wilson 2004; Moss et al. 2006; Bunnell et al. 2011; Desouza et al. 2012). However, the functional significance of interactions between the BIN1 tumor suppressor and these cytoskeletal proteins in CTCL cells is still elusive and further investigations are required. In addition, I also assessed the effect of AH1-1 on methylation status of the BIN1 basal promoter, by means of bisulfite sequencing. It was shown that BIN1 is mostly unmethylated and differential expression of AH1-1 has no significant effect on methylation status of the BIN1 basal promoter in Hut78 cells, although additional studies will
be needed to study the rest of the regulatory regions of BIN1. In addition, I investigated the effect of BIN1 overexpression on cell proliferation and apoptosis in AHI-1-suppressed cells. A significant decrease in the CFC output and a significant increase in the number of apoptotic cells were observed in AHI-1-suppressed cells compared to parental Hut78 cells. However, no additional differences were observed in the CFC output or apoptosis after overexpression of BIN1 in AHI-1-suppressed cells, possibly due to the high endogenous levels of BIN1 in these cells.
Figure 5.1: Model for BIN1 function in regulation of the Fas/FasL-mediated apoptosis through c-FLIP in CTCL

Model of how deregulated expression of BIN1 isoforms regulates the Fas/FasL–mediated apoptosis through c-FLIP in CTCL. The loss of tumor suppressor activity of BIN1 contributes to enhanced proliferation and apoptosis resistance of CTCL cells through upregulation of c-FLIP, which mediates activation of the caspase cascade and enhances transforming activity of these cells in mice.
5.2 Significance and limitations of the Work

To my knowledge, this is the first study to explore the functions of BIN1 and its isoforms in a hematopoietic malignancy. This study provides the first evidence of strong tumor suppressor activity of two BIN1 isoforms, BIN1 (+10, +13) and BIN1 (+10, +12A, +13), in primary CTCL cells and cell lines by means of *in vitro* and *in vivo* assays.

Because of the low proliferation rate of CTCL cells, it has been proposed that their accumulation may, to some extent, be due to defects in apoptosis pathways. In relation to these properties of CTCL cells, patients with CTCL are usually resistant to chemotherapy that targets rapidly dividing cells (Dummer *et al.* 1995; Braun *et al.* 2007; Contassot *et al.* 2008; Hwang *et al.* 2008; Wang *et al.* 2011). Thus, identifying mechanisms involved in the development of apoptosis resistance in CTCL cells has become one of the major aspects of CTCL research, in the hopes of developing new therapeutic targets. In this study, I have demonstrated the downregulation of BIN1 and subsequent upregulation of c-FLIP as a mechanism for developing apoptosis resistance in CTCL cells. Importantly, I have further demonstrated that restored expression of BIN1 makes these cells more sensitive to Fas/FasL-induced apoptosis. Thus, this study provides new evidence that the loss of tumor suppressor activity of BIN1 regulates the Fas/FasL-induced apoptosis through c-FLIP by causing apoptosis resistance in CTCL cells. These findings further propose BIN1 and c-FLIP as potential therapeutic targets in CTCL. Interestingly, elevated c-FLIP levels have been reported in various tumor types (Valente *et al.* 2006; El-Gazzar *et al.* 2010; Haag *et al.* 2011) and targeting c-FLIP in combination with other chemotherapy agents have been suggested to be an effective strategy to conquer drug resistance in cancer cells (Safa *et al.* 2008; Safa & Pollok 2011). However, the significant structural similarity between c-FLIP and caspase 8, makes the c-FLIP protein a difficult target for drugs to
inhibit its function. Small molecule ligands that are capable of blocking the recruitment of c-FLIP to the DISC, could also inhibit the recruitment of caspase 8 and thus prevent the initiation of apoptosis. As a result, it is needed to develop small molecules that inhibit the c-FLIP function without targeting caspase 8 (Safa & Pollok 2011; Safa 2012). Nevertheless, several studies have shown that inhibition of c-FLIP transcription or translation with the use of liposomal complexes of c-FLIP-specific siRNA or antisense phosphorothioate oligonucleotide can significantly increase apoptosis in malignant cells in vitro and in vivo (Day et al. 2006; Day et al. 2008; Day et al. 2009; Logan et al. 2010). The development of Oligonucleotide and RNAi-targeting of c-FLIP for cancer therapy hold promise for improving the way cancers, including CTCL, are treated. However, a crucial first step is to resolve the difficulties in siRNA design, delivery and stability (Safa et al. 2008; Safa 2012). In addition to c-FLIP, this study also suggests that restoring BIN1 expression in CTCL cells may represent another effective strategy for overcoming apoptosis resistance, leading to more effective therapies for CTCL.

In addition to results generated by in vitro and in vivo assays, I further confirmed the importance of BIN1 and c-FLIP in CTCL by demonstrating a significant downregulation of BIN1 and a significant upregulation of c-FLIP transcripts in MF and SS patient samples. Also, the correlation between the high BIN1/ low c-FLIP mRNA levels and a better survival rate in patients with SS, further point out to the importance of these two proteins in CTCL pathogenesis and disease outcome. However, CTCL is a rare disease and as a result, one of the limitations of the current study was the limited number of primary MF and SS samples. In total, expression levels of BIN1 and c-FLIP were assessed in 15 MF and 13 SS samples and compared to maximum 6 controls with BID. The correlation study was conducted on only 12 SS samples. A larger sample size increases the power of the study, is more representative of the population, and
minimizes the influence of outliers or extreme observations. Therefore, it would be useful to further measure the expression levels of BIN1, as well as c-FLIP in a larger CTCL sample size, if these samples are available. Furthermore, having access to a larger CTCL sample size will give us the opportunity to further investigate the association between BIN1 or c-FLIP expression levels and their prognostic impact on CTCL patients.

5.3 Future Directions

Although I have demonstrated that differential expression of BIN1 mediates expression changes of c-FLIP in CTCL cells, the mechanism underlying this association has remained unexplored. c-FLIP expression can be regulated at multiple levels. At the transcriptional level, c-FLIP expression can be modulated by a variety of transcription factors including NF-κB, p63, E2F1 and c-MYC (Safa et al. 2008; Safa 2012). Interestingly, it has been reported that the nuclear-localized isoforms of BIN1 can physically interact with c-MYC and inhibit the transactivation of several c-MYC target genes (Elliott et al. 1999). In this study, I have shown that restoration of BIN1 leads to a significant downregulation of c-FLIP at both the mRNA and protein levels. Therefore, based on our observation and the previous reports on the function of BIN1 as a transactivation inhibitor, it is of interest to further explore whether BIN1 has any effect on the transactivation of the c-FLIP promoter, using a luciferase reporter assay.

Furthermore, c-FLIP protein expression and turnover is tightly regulated post-translationally by the ubiquitin-proteasome degradation system (Safa et al. 2008; Bagnoli et al. 2010; Safa 2012). One study has shown that activation of Jun kinase leads to phosphorylation and activation of the E3 ubiquitin ligase Itch, which causes ubiquitination and degradation of the
c-FLIP protein in mouse hepatocytes (Chang et al. 2006). Another study on human
erythroleukemia cells (K562), has demonstrated that phosphorylation of c-FLIP at the serine 193
by protein kinase C, opposes ubiquitylation and stabilizes c-FLIP levels (Kaunisto et al. 2009).
In this study, I showed that overexpression of BIN1 leads to downregulation of c-FLIP and
further suppression of BIN1 enhances the c-FLIP protein levels. These results suggest that BIN1
may have negative effects on the stability of the c-FLIP protein. To test this hypothesis, the
amount of ubiquitinated c-FLIP can be measured in the presence and absence of BIN1.

Moreover, as described in Section 1.3.3, miRNAs play important roles in CTCL
pathogenesis and they may also be useful in CTCL diagnosis. Interestingly, it has been
demonstrated that miR-512-3p binds to the 3' untranslated region of c-FLIP and negatively
regulates its expression in HepG2 hepatocellular carcinoma cells (Chen et al. 2010).
Transfection of miR-512-3p in HepG2 cells downregulates the c-FLIP protein levels and
significantly enhances the taxol-induced apoptosis (Chen et al. 2010). It would be interesting to
further investigate the expression level of miR-512-3p and its potential correlation to c-FLIP
levels in CTCL cell lines and patient samples. Furthermore, additional putative miRNAs that
may regulate the c-FLIP and BIN1 expression levels in CTCL cells can be identified through
bioinformatics analyses and their roles in CTCL pathogenesis, diagnosis and/or prognosis can be
further assessed using biological assays. All together, these experiments will provide more
information about the BIN1/c-FLIP pathway in CTCL cells and thus may lead to a deeper insight
into the roles of specific proteins and their signaling pathways in CTCL development.

As discussed earlier, CTCL cells are usually resistant to chemotherapy due to defects in
their apoptosis pathways. Several studies have previously reported the role of c-FLIP and BIN1
in regulating the sensitivity of different cancer cells to several anti-cancer agents and
chemotherapy drugs (Day et al. 2008; Zhong et al. 2009; Logan et al. 2010; Pyndiah et al. 2011; Tanida et al. 2012). In this study, I have demonstrated that the loss of BIN1 and subsequent upregulation of c-FLIP induce apoptosis resistance in CTCL cell lines. Interestingly, based on some preliminary experiments, I have recently observed that restored expression of BIN1 in CTCL cell lines enhances their sensitivity to several chemotherapy drugs including doxorubicin (Appendix Figure A.4). Doxorubicin is among the most suitable chemotherapy options for the treatment of advanced stages of CTCL (Wollina et al. 2003; Assaf et al. 2013). However, more in vitro and in vivo biological experiments, including manipulation of expression of BIN1 and c-FLIP, their isoforms and mutants, are required to elucidate molecular and cellular functions of these two proteins in the regulation of chemosensitivity of CTCL cells. It is strongly believed that more effective treatment strategies will be developed by continually identifying new therapeutic targets and by the introduction of combination regimens of chemotherapy and targeted therapy (Safa et al. 2008).

In addition, further experiments are required to identify a potential biological link between BIN1 and AHI-1 in CTCL cells. Preliminary results have shown that differential expression of BIN1 has no effect on the AHI-1 expression levels in Hut78 cells, using western blot analysis (Appendix Figure A.5). In contrast, previous members of our group and I have demonstrated that differential expression of AHI-1 manipulates the BIN1 expression levels in CTCL cells. These results suggest that AHI-1 and BIN1 are involved in the same signaling pathways and AHI-1 acts upstream of BIN1. Further experiments conducted in BIN1- and AHI-1-overexpressed and suppressed CTCL cells (Hut78 and HH) are needed to further confirm this observation. Furthermore, I have shown that BIN1 is involved in the Fas/FasL-mediated apoptosis pathway in CTCL cells and regulates the c-FLIP expression levels. To assess whether
AHI-1 is also involved in this apoptosis pathway, AHI-1-suppressed and AHI-1-overexpressed CTCL cells (Hut78 and HH) can be used to further investigate the effects of differential expression levels of AHI-1 on BIN1 and different components of the Fas/FasL-mediated apoptosis pathway, including c-FLIP, caspase 8 and caspase 3. These experiments may provide useful information regarding the potential biological association between BIN1 and AHI-1 and their possible involvement in the same signaling pathways in CTCL cells.

Ultimately, these proposed studies will lead to better understanding of the underlying mechanisms involved in CTCL pathogenesis. A deeper insight into the roles of specific genes and signaling pathways involved in CTCL development and pathogenesis will lead to more effective treatment options.
References


Figure A.1: Restored BIN1 expression significantly decreases the transforming activity of CTCL cells in vivo

(A) Tumor volume comparison between NOD/SCID mice injected with BIN1-transduced HH cells containing two isoforms (n=6 for HHBIN1 and n=6 for HH12A) and controls (n=3 for HH cells and n=2 for HHMPG vector as positive controls; n=1 injected with PBS as negative controls). Mean tumor volumes ± SEM are shown. (B) Representative images of dissected large tumors from HHMPG mice versus smaller tumors from HHBIN1 and HH12A mice, around 4 weeks post-injection. (C) Comparison of tumor weights in HH/HHMPG mice versus HHBIN1 and HH12A mice about 4 weeks post-injection.
**Figure A.2:** Restored BIN1 expression in Hut78 cells does not affect the c-MYC protein expression levels

Western blot analysis of c-MYC in BIN1-transduced Hut78 cells. HutMPG = vector control-transduced cells, HutBIN1 = BIN1 (+10, +13)-transduced cells and Hut12A = BIN1 (+10, +12A, +13)-transduced cells. GAPDH was used as a control for protein loading.

**Figure A.3:** Western blot analysis of AHI-1 in Hut78 cells

Protein expression levels of AHI-1 in parental Hut78, empty vector control Hut78 (RPG), AHI-1-suppressed Hut78 (AHI-1-sh4-1 and AHI-1-sh4-2) and AHI-1-overexpressed Hut78 (Lenti-AHI-1) cells. GAPDH was used as a control for protein loading.
Figure A.4: Restored BIN1 expression increases the chemosensitivity of CTCL cells

Percentage of viable cells determined by trypan blue exclusion assay in BIN1-transduced Hut78 cells (left) and BIN1-transduced HH cells (right) after treating the cells for 24 hours with different dosage of doxorubicin.

Figure A.5: Western blot analysis of AHI-1 in BIN1-transduced Hut78 and BIN1-transduced HH cells

Left panel: protein expression levels of AHI-1 in parental Hut78, empty vector control Hut78 (HutMPG) and BIN1-transduced Hut78 cells (HutBIN1 and Hut12A). Right panel: protein expression levels of AHI-1 in empty vector control HH (HHMPG) and BIN1-transduced HH cells (HHBIN1 and HH12A). GAPDH was used as a control for protein loading.
Table A.1: Clinical features of patients with Mycosis Fungoides (MF, n=15).

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Patient Description</th>
<th>Age</th>
<th>Sex</th>
<th>Origin</th>
<th>Genes evaluated by q-RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF-1</td>
<td>MF patient, Stage I (Patch)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Total BIN1, BIN1 (+12A)</td>
</tr>
<tr>
<td>MF-2</td>
<td>MF patient, Stage I (Patch)</td>
<td>42</td>
<td>F</td>
<td>Chinese</td>
<td>Total BIN1, BIN1 (+12A)</td>
</tr>
<tr>
<td>MF-3</td>
<td>MF patient, Stage I (Plaque)</td>
<td>46</td>
<td>M</td>
<td>Caucasian</td>
<td>Total BIN1, BIN1 (+12A)</td>
</tr>
<tr>
<td>MF-4</td>
<td>MF Patient, Stage I (Plaque)</td>
<td>46</td>
<td>F</td>
<td>Caucasian</td>
<td>Total BIN1, BIN1 (+12A), c-FLIP</td>
</tr>
<tr>
<td>MF-5</td>
<td>MF Patient, Stage I (Plaque)</td>
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<td>Caucasian</td>
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</tr>
<tr>
<td>MF-6</td>
<td>MF Patient, Stage IV (Plaque)</td>
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<td>Caucasian</td>
<td>Total BIN1, BIN1 (+12A), c-FLIP</td>
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<td>M</td>
<td>Caucasian</td>
<td>Total BIN1, BIN1 (+12A)</td>
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<tr>
<td>MF-8</td>
<td>MF Patient, Stage II (Plaque)</td>
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<td>Caucasian</td>
<td>Total BIN1, BIN1 (+12A)</td>
</tr>
<tr>
<td>MF-9</td>
<td>MF Patient, Stage I (Plaque)</td>
<td>40</td>
<td>F</td>
<td>Asian</td>
<td>Total BIN1, BIN1 (+12A)</td>
</tr>
<tr>
<td>MF-10</td>
<td>MF Patient, Stage II (Plaque)</td>
<td>40</td>
<td>M</td>
<td>Indian</td>
<td>Total BIN1, BIN1 (+12A), c-FLIP</td>
</tr>
<tr>
<td>MF-11</td>
<td>MF Patient, Stage I (Patch)</td>
<td>60</td>
<td>M</td>
<td>Caucasian</td>
<td>c-FLIP</td>
</tr>
<tr>
<td>MF-12</td>
<td>MF Patient, stage III (Tumor)</td>
<td>54</td>
<td>M</td>
<td>Chinese</td>
<td>c-FLIP</td>
</tr>
<tr>
<td>MF-13</td>
<td>MF Patient, stage III (Tumor)</td>
<td>54</td>
<td>M</td>
<td>Chinese</td>
<td>c-FLIP</td>
</tr>
<tr>
<td>MF-14</td>
<td>MF Patient, Stage I (Plaque)</td>
<td>85</td>
<td>F</td>
<td>Asian</td>
<td>c-FLIP</td>
</tr>
<tr>
<td>MF-15</td>
<td>MF Patient, stage I (Patch)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>c-FLIP</td>
</tr>
</tbody>
</table>

NA indicates information not available.
Table A.2: Clinical features of patients with Sezary syndrome (SS, n=13)

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Age</th>
<th>Sex</th>
<th>Death Status</th>
<th>TCR clonality</th>
<th>Duration of Survival (Months)</th>
<th>Sezary cells (%)</th>
<th>CD4+ cells (%)</th>
<th>CD4+ CD7- cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS1*</td>
<td>78</td>
<td>M</td>
<td>Dead</td>
<td>Yes</td>
<td>75</td>
<td>&gt;5%</td>
<td>95%</td>
<td>94%</td>
</tr>
<tr>
<td>SS2</td>
<td>74</td>
<td>F</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>&gt;5%</td>
<td>88%</td>
<td>85%</td>
</tr>
<tr>
<td>SS3*</td>
<td>73</td>
<td>M</td>
<td>Dead</td>
<td>No</td>
<td>120</td>
<td>&lt;5%</td>
<td>54%</td>
<td>27%</td>
</tr>
<tr>
<td>SS4*</td>
<td>66</td>
<td>F</td>
<td>Alive</td>
<td>No</td>
<td>152</td>
<td>&gt;5%</td>
<td>94%</td>
<td>84%</td>
</tr>
<tr>
<td>SS5*</td>
<td>78</td>
<td>M</td>
<td>Dead</td>
<td>Yes</td>
<td>47</td>
<td>&gt;5%</td>
<td>95%</td>
<td>94%</td>
</tr>
<tr>
<td>SS6*</td>
<td>59</td>
<td>M</td>
<td>Dead</td>
<td>No</td>
<td>51</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SS7*</td>
<td>77</td>
<td>F</td>
<td>Dead</td>
<td>No</td>
<td>14</td>
<td>&gt;5%</td>
<td>88%</td>
<td>80%</td>
</tr>
<tr>
<td>SS8*</td>
<td>52</td>
<td>M</td>
<td>Dead</td>
<td>Yes</td>
<td>34</td>
<td>&gt;5%</td>
<td>85%</td>
<td>3%</td>
</tr>
<tr>
<td>SS9*</td>
<td>82</td>
<td>F</td>
<td>Dead</td>
<td>Yes</td>
<td>27</td>
<td>&gt;5%</td>
<td>80%</td>
<td>68%</td>
</tr>
<tr>
<td>SS10*</td>
<td>74</td>
<td>F</td>
<td>Dead</td>
<td>No</td>
<td>20</td>
<td>&gt;75%</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SS11*</td>
<td>60</td>
<td>M</td>
<td>Dead</td>
<td>Yes</td>
<td>6</td>
<td>&gt;5%</td>
<td>95%</td>
<td>79%</td>
</tr>
<tr>
<td>SS12*</td>
<td>43</td>
<td>F</td>
<td>Alive</td>
<td>Yes</td>
<td>31</td>
<td>&gt;5%</td>
<td>NA</td>
<td>68%</td>
</tr>
<tr>
<td>SS13*</td>
<td>61</td>
<td>F</td>
<td>Alive</td>
<td>Yes</td>
<td>28</td>
<td>&gt;5%</td>
<td>86%</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA indicates information not available.

* Indicates patients that were included in the Kaplan-meier survival analysis.